ANALYTICAL METHODS IN THE FOOD INDUSTRY

A collection of the papers presented at the Symposium on Analytical Methods in the Food Industry held by the Divisions of Analytical Chemistry and Agricultural and Food Chemistry of the American Chemical Society at the 115th national meeting in San Francisco, March 28 to April 1, 1949

Number three of the Advances in Chemistry Series
Edited by the staff of *Industrial and Engineering Chemistry*

Published September 13, 1950, by
AMERICAN CHEMICAL SOCIETY
1155 Sixteenth Street, N.W.
Washington, D. C.

Introduction

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch001

Among the spectacular scientific accomplishments of the historically recent past, none has made a more profound contribution to our physical well-being than have those of Appert, Pasteur, and others, through which we have gained practical ascendancy over the world of food spoilage microorganisms. Shorn of the safeguards founded firmly on those researches, modern civilization, if possible at all, would be quite different, and many of our common foods would be unknown. In any event, mastery of the basic principles of spoilage prevention has permitted turning our scientific searchlight on the quality of our daily fare and it is here, of course, that the techniques of food analysis make their indispensable contribution.

Why should we wish to know the composition of foods?

Perhaps, first of all, we must know that our food is nutritious, that it contains the elements essential to growth and maintenance of our bodies in optimum amount along with the calories needed for the fuel supply. As our living habits become more complex, we are increasingly dependent on precise analysis because the naturally balanced diet of our ancestors is no longer to be had by most of us.

Second only to its adequacy, our food must be wholesome and our very existence bespeaks the excellent job our food-analyst guardians are doing to ensure that we receive exactly what we bargain for—that is, clean, unspoiled food, unadulterated with any undeclared substance, harmful or otherwise.

Thirdly, the research worker in countless fields must depend on the methods of food analysis for control of his experiments, and this can be vital. It has been pointed out recently, for example, that the observed toxicity of certain substances may be affected significantly by the composition of the basic diet.

Opportunities for Food Research

Perhaps to the food technologist, food analysis is most important of all, for to him it provides means for assessing the quality of his product. He must know not only that the food he prepares is nutritionally sufficient and that it is clean and unadulterated, but also that it is good to eat. In no field of food research does so much remain to be learned. What are the substances responsible for the characteristic flavors of foods? We know a few of the simpler ones, but the chemistry of our common fruit and vegetable flavors is almost wholly unexplored. Even when known, their analysis will not prove simple, for it is readily apparent that they are very complex mixtures. Our knowledge of food colors is somewhat more advanced than in the case of flavors. The chemistry of many of the important pigments is known and we can at least describe with confidence the colors of many clear liquid foods; maple sirup is an example. For many years the measurement of texture of food products has merited and received a great deal of study. As a result a few simple measurements can be made and reproduced. The toughness of meat and the tenderness of raw, if not of cooked peas, can be determined; but very little is known of the

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chemical factors that affect texture. What, for instance, determines the moisture relationships within foods, and how does it change on cooking or processing or storage?

The researcher in food and its analysis is keenly aware that his task will not be finished until the "quality" of a food product can be defined completely in precise terms of its flavor, color, texture, and nutritive value. The goal is distant but the journey is well begun. The papers contained herein describe the present state of affairs in each of as many of the fields of food analysis as time for the symposium permitted. Each has been covered by an outstanding worker in his field. It is unfortunate that B. L. Oser's excellent paper on "Advances in Vitamin Determination" does not appear. His more comprehensive review of food analysis which appeared in *Analytical Chemistry* [**21,** 216 (1949)] should by all means be studied along with the papers contained herein.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch001

Measurement of Color Changes in Foods

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Methods are described for determining the extent to which original natural color is preserved in processing and subsequent storage of foods. Color differences may be evaluated indirectly in terms of some physical characteristic of the sample or extracted fraction thereof that is largely responsible for the color characteristics. For evaluation more directly in terms of what the observer actually sees, color differences are measured by reflectance spectrophotometry and photoelectric colorimetry and expressed as differences in psychophysical indexes such as luminous reflectance and chromaticity. The reflectance spectrophotometric method provides time-constant records in research investigation on foods, while photoelectric colorimeters and reflectometers may prove useful in industrial color applications. Psychophysical notation may be converted by standard methods to the colorimetrically more descriptive terms of Munsell hue, value, and chroma. Here color charts are useful for a direct evaluation of results.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch002

Color is a significant factor in the consumer acceptability of foods. The consumer's reaction may be simple dislike for a certain color or, more likely, a reaction based on association of certain color characteristics with fresh and wholesome quality. More fundamental is the fact that color is often directly related to nutritive factors such as carotene (nutritionally important as provitamin A). Some degree of correlation has been found between color and general quality in certain industrial products such as vegetable oils, but the problem is more complicated with fresh and processed foods. Regardless of the degree to which color is a true indication of palatability or nutritional quality, it is a very evident characteristic of foods and is recognized as important in quality grading. Many quality standards, including a color factor, have already been officially established. Fresh and processed fruits and vegetables, fats and oils, meats, dairy products, poultry, and eggs are among the foods in which color is important in quality standards.

Factors affecting the color of foods include hereditary varietal differences, maturity, growing conditions (temperature, moisture, locality), and processing procedures. The first three operate in a complex way on the raw product and result in an original natural color over which the food processor has control only in so far as he can select his raw material. However, the extent to which this original natural color is preserved during processing and in subsequent storage is one important criterion of processing procedures. This discussion is devoted to some of the methods that may be used to characterize differences in natural color of food products and to detect and specify changes in reflection or absorption characteristics that occur as a result of processing treatment and storage conditions, even though no associated change in visual color is perceptible.

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Measurement of Physical Characteristics Related to Color

The objective indication of color differences in foods has usually been attempted in a simplified, indirect way that involves a comparison of some physical characteristic of the samples or, more often, an extracted fraction that is assumed or has been proved to be largely responsible for the associated color characteristics. Although such a method does not measure the actual visual color of the samples, a measure of relative amounts of color-characteristic pigments or a comparison of physical properties of extracts of color-critical fractions (which may be mixtures of several pigments) may prove to be very sensitive indications of differences that are closely related to color.

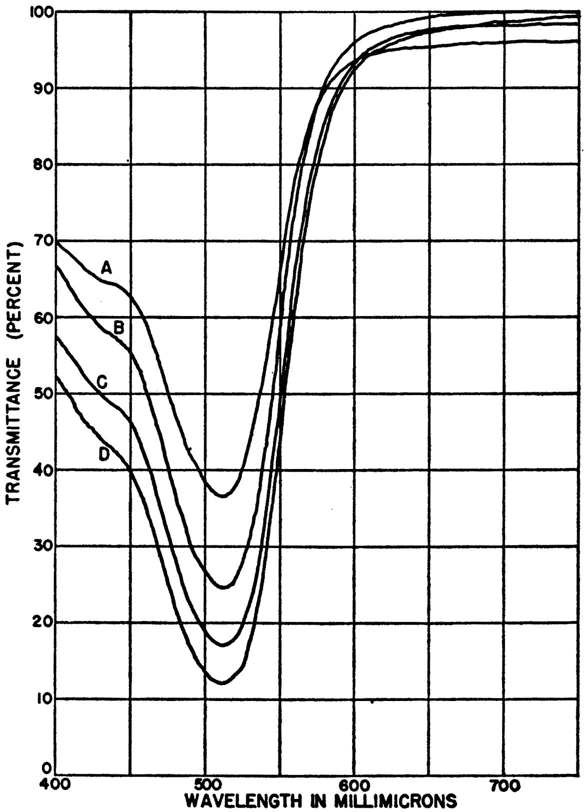


Figure 1. Varietal Differences in Raspberries as Indicated by Transmittance of Centrifuged Juices

A. Newburg
B. Tahoma
C. Cuthbert
D. Willamette

Spectrophotometry. The instrument generally used for this basic type of measurement is the spectrophotometer. The data obtained, usually pictured in the form of a spectrophotometric curve, indicate the ability of the sample to transmit or reflect light of the various wave lengths. Various instruments are available which can be used to determine more or less complete spectrophotometric curves.

The important thing about such a spectrophotometric curve is that it describes a physical property of the material that is fundamentally related to its color. If, then, the color-determining component can be extracted from the product under test, a

transmittance spectrophotometric measurement is descriptive of this fraction and differences in the spectrophotometric properties of such fractions from separate samples are thus indirectly indicative of possible color differences in the samples. Such methods have been used in the study of tomato color (*7*) and color change in green vegetables (*8*). Kramer and Smith (*6*) have used spectrophotometric indexes of extracted color fractions in the study of color differences in various foods.

Such a method has been used to indicate differences between varieties of raspberries (Figure 1). Samples were blended and centrifuged for 15 to 20 minutes at 2000 r.p.m. in 100-ml. tubes. The clear juice was pipetted off and diluted with 9 parts of water. The pH was adjusted to that of the original undiluted juice, and transmittance curves were run for samples in 2.5-cm. cells. The differences between the varieties are apparent from the curves.

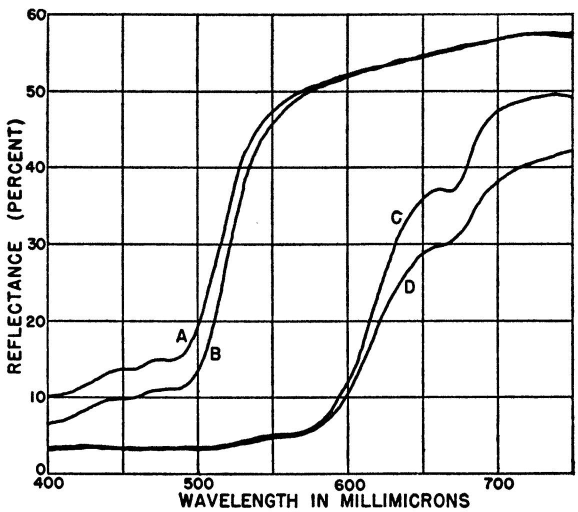


Figure 2. Color Changes Indicated by Reflectance Spectrophotometry

A. Yellow sweet corn (whole grain)
B. More mature corn
C. Normally processed
D. Heat-damaged tomato paste

In a similar way, reflectance spectrophotometry has been used to indicate related color changes in certain foods. Figure 2 shows differences in the reflectance characteristics of yellow sweet corn (whole grain) of two different maturities, and properly processed tomato paste and paste damaged by overheating. As an additional example, Figure 3 shows the striking differences in the surface reflectance of lemons of different color grades. (Colorimetric calculations which could be made on the basis of the curves of Figures 1 to 3 to evaluate the color more directly in terms of what an observer sees are described in a later section.)

Abridged Spectrophotometry. It is not always necessary to obtain complete spectrophotometric curves in order to measure physical characteristics related to color. The procedure can often be considerably simplified by some abridged form of spectrophotometry. Measurements may be made only at critical wave lengths or wave-length bands, as has been done to determine chlorophyll degradation (*1*, *8*). In such instances the real problem that faces the investigator is to establish the critical wave lengths.

Such a simplification could be carried out in the example cited above for raspberries, where a transmittance measurement in the region of maximum absorption

(around 510 mμ) could be used as an index of color difference in the extracts. A simple filter colorimeter would probably be satisfactory for such a purpose. Similarly, an instrument capable of measuring reflectance at a specific wave length or band of wave lengths could be used to detect the differences in corn and tomato paste cited. As the corn matures, the apparent increase in yellow color results more from a decrease in blue reflectance than from an increase in yellow, and in this particular instance the change in blue reflectance is a more sensitive index than the over-all color change.

When the interest is in acceptability of visual color, the use of such indirect indexes in substitution for the color of the product depends upon how well the index is related to the color characteristics of the original product. While the actual measurement of the transmittance index may be more precise than the reflectance index, chiefly because of sampling difficulties, it must be established that the color of the extract represents the total color of the original product. In abridged transmission methods the extracted fraction, in addition to being representative of color change, must also be simple and pure enough that change in a specific region is indicative of total color change. These conditions are only rarely satisfied in studying color of processed food systems. As might be expected, certain fractions influencing color may be difficult to remove or may not be removed by the extraction method used, and color changes which occur in these nonextracted pigments would not be included in the transmittance measurements. Because the visual color of a food product depends upon its reflectance characteristics, total color differences can be studied by reflectance spectrophotometry and colorimetry.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch002

Psychophysical Methods for Measurement and Designation of Reflectance Color in Foods

The indirect methods discussed thus far have dealt with measurement of color only as it can be correlated with physical characteristics of materials and the effect of these materials on radiant energy. As has been pointed out, the reflectance spectrophotometric curve describes a property of the material. A change in the reflectance spectrophotometric properties may not always result in a change in visual color. The reason is that "color of the object" is not an unchangeable characteristic of the object itself, dependent only upon these reflectance properties, but is also dependent upon the quality of the illuminating light and the sensitivity of the observer's eye. Thus the measurement and description of visual color are psychophysical problems (*14*).

Subjective Description of Color in Terms of Equivalent Stimuli. The observer, unable directly to measure or describe a color sensation in absolute terms, is able to evaluate it in terms of certain stimuli which produce an equivalent sensation. Subjectively the comparison is accomplished experimentally with a "colorimeter," so designed that the color of the sample is seen in one half of a photometric field and the "mixture" of color produced by independently controllable components is seen in the other half. By proper adjustment of the components, a unique setting will be found which produces a match in the photometric field and the color of the sample can be specified in terms of the amounts of the chosen components.

One method for subjective evaluation of the surface color of foods in terms of equivalent stimuli is accomplished by the method of "disk colorimetry" (*12*). The color of a sample is matched by proper adjustment of a set of radially slit colored disks, the light from which is mixed by rotating the disks. Some instruments are equipped with a revolving optical mechanism for mixing the light from the disks, and because the disks themselves thus remain stationary, adjustments can be made while the machine is in operation. A set of disks is chosen depending on the product and the range of color to be measured. Usually one set of four colors can be selected to cover the entire color range for a particular commodity. The set chosen for green peas will obviously differ from that chosen for tomatoes. The result of the color match is expressed by a record of the relative amounts of the disks necessary for a

match. Such a method has been used by Kramer and Smith (*6*) in measuring the color of various foods.

The method, obviously, is subjective, the precision and speed of the match depending upon the observer and his experience. Results on foods have usually been expressed in terms of color disks, which are different for each product and which must be carefully standardized. [Conversions to standard colorimetric systems of notation can be made (*12*), provided suitable colorimetric data are available for the disks used.] Furthermore, instruments suitable for the most precise work by this method are not at the present time commercially available.

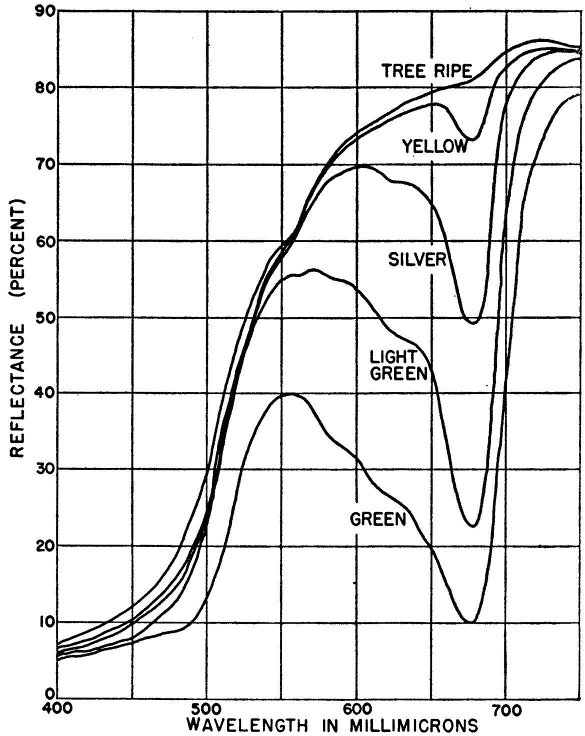


Figure 3. Surface Reflectance of Lemons from Five Different Color Grades

Objective Evaluation of Color. In recent years a method has been devised and internationally adopted (International Commission on Illumination, I.C.I.) that makes possible objective specification of color in terms of equivalent stimuli. It provides a common language for description of the color of an object illuminated by a standard illuminant and viewed by a "standard observer" (*14*). Reflectance spectrophotometric curves, such as those described above, provide the necessary data. The results are expressed in one of two systems: the tristimulus system in which the equivalent stimulus is a mixture of three standard primaries, or the heterogeneous-homogeneous system in which the equivalent stimulus is a mixture of light from a standard heterogeneous illuminant and a pure spectrum color (dominant wave-length–purity system). These systems provide a means of expressing the objective time-constant spectrophotometric results in numerical form, more suitable for tabulation and correlation studies. In the application to food work, the necessary experimental data have been obtained with spectrophotometers or certain photoelectric colorimeters.

Spectrophotometric Method. The spectrophotometric curves of the various

foods studied were obtained with a Hardy recording spectrophotometer (*2, 9*). The I.C.I. tristimulus values (X, Y, and Z) were obtained by integration of these curves by standard methods (*1*). The trichromatic coefficients, x and y, were calculated and dominant wave length and excitation purity were read from large scale chromaticity charts (*1*).

The experimental problems are typical of measurements on agricultural material. Many types of samples are encountered—powders, diced dried vegetables, sliced and pureed foods, frozen whole vegetables, etc.—each giving rise to problems of sampling, preparation, presentation in the instrument, etc. Total color difference over the range of otherwise acceptable samples is usually small and thus requires considerable precision of measurement. Color changes may take place very rapidly and thus samples must be treated and measured quickly, as illustrated in Figure 4, which shows the rate of browning of frozen peaches after thawing to room temperature.

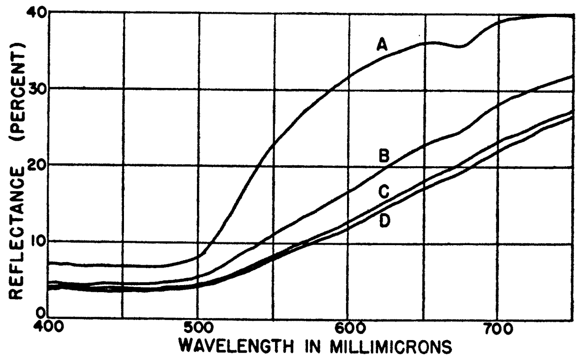


Figure 4. Variation in Reflectance of Frozen Peaches with Time after Thawing

A. Immediately after thawing
B. After 90 minutes
C. After 180 minutes
D. After 270 minutes

In this spectrophotometer the sample must be placed behind a vertical window. This condition is met either by pressing the sample into a block, which is feasible only when the moisture content is right, or by placing it in a flat glass cell. The cell should be of sufficient thickness to prevent introduction of interferences by reflections off the backing or cell support.

Sample preparation is complicated by the variety of forms encountered. Homogenization, by grinding or pulping, may or may not be allowable in accordance with the purpose of the investigation. In consumer acceptability studies, blending destroys the significance of the result as far as surface color is concerned, and the sample is studied in its actual form whenever possible. When color is used as an analytical index of change during processing or storage, blending may be permissible and may be necessary to give sufficient precision to results. Blending may be necessary for other reasons, as in comparison of products that may or may not become broken up in processing or that may be processed in different forms such as dice or slices. Obviously, blending is not allowable at all when the purpose of the investigation involves variation of color from place to place within the sample itself.

Marked changes occur in the visible appearance of dehydrated foods with variation in particle size. It has been found that this effect is chiefly one of variation in luminous reflectance, Y (see Tables I and II). In some instances (note the data for cabbage), chromaticity (x, y) remains so nearly constant over a fairly wide range of particle size that it appears possible that for certain products and purposes the effect of particle size might be eliminated by the choice of chromaticity as a color variable.

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Table I. Color Variations in Certain Dehydrated Foods with Variation of Particle Size

Product	Mesh Size	Y	x	y
Cabbage	Unground	0.316	0.358	0.381
	10–18	0.380	0.357	0.378
	24–35	0.396	0.356	0.377
	60–80	0.440	0.351	0.375
	100–120	0.509	0.347	0.370
Carrots (diced)	Unground	0.170	0.380	0.352
	10–18	0.173	0.413	0.361
	24–35	0.252	0.416	0.373
	60–80	0.321	0.436	0.395
	100–120	0.397	0.423	0.396

In the more usual case, however, if small differences are to be measured, it is likely that particle size will have to be standardized. Generally speaking, differences between two unlike samples are more apparent visually for samples of larger particle size (see Table II). Often samples noticeably different in diced form are practically indistinguishable if ground to a very fine powder.

The application of reflectance spectrophotometry in studying color changes in foods is illustrated by an experiment in which five samples of peas were held in the pod at room temperature—that is, under market conditions—for various periods of time before cooking. Measurements were made on samples podded and cooked and the whole peas packed in flat glass cells. The cells were filled with water to cut down specular reflection from the curved surfaces of the peas. The resulting spectrophotometric curves are shown in Figure 5. The I.C.I. data obtained from these curves are given in Table III. It is immediately apparent from the curves that there is an increase in luminous reflectance—i.e., the color of the peas becomes lighter—with delay before cooking. There is also some trend toward longer dominant wave length (yellower hue) apparent in the numerical data.

Photoelectric-Colorimetric Method. Although the recording spectrophotometer is, for food work at least, a research tool, another instrument, the Hunter multipurpose reflectometer (*4*), is available and may prove to be applicable to industrial quality control. (The newer Hunter color and color difference meter which eliminates considerable calculation will probably be even more directly applicable. Another make of reflection meter has recently been made available commercially that uses filters similar to those developed by Hunter and can be used to obtain a similar type of data.) This instrument is not a spectrophotometer, for it does not primarily measure the variation of any property of samples with respect to wave length, but certain colorimetric indexes are calculated from separate readings with amber, blue, and green filters, designated *A*, *B*, and *G*, respectively. The most useful indexes in food color work obtainable with this type of instrument have been *G*, which gives a

Table II. Effect of Particle Size on Apparent Color Difference between Dissimilar Samples of Dehydrated Potatoes

Sample	Y	x	y	λ, mμ	p, %	Munsell Notation Hue	Munsell Notation Value/chroma
Particle Size, 1 to 2 Mm.							
a	0.563	0.374	0.386	576.4	35.3	3.8 Y	7.84/4.6
b	0.470	0.393	0.386	580.0	41.0	0.4 Y	7.26/5.2
c	0.340	0.376	0.369	580.5	31.8	9.8 YR	6.33/3.6
Particle Size, 0.5 to 1 Mm.							
a	0.605	0.369	0.383	575.9	33.9	4.1 Y	8.08/4.3
b	0.524	0.385	0.384	579.0	38.1	1.4 Y	7.61/5.0
c	0.390	0.371	0.368	579.8	30.2	0.4 Y	6.71/3.5
Particle Size, 0.25 to 0.5 Mm.							
a	0.699	0.356	0.369	575.7	26.2	4.2 Y	8.58/3.4
b	0.628	0.368	0.373	577.8	30.4	1.9 Y	8.21/4.0
c	0.494	0.361	0.363	578.7	26.0	1.3 Y	7.42/3.2
Particle Size, 0.125 to 0.25 Mm.							
a	0.791	0.336	0.344	576.3	14.4	2.5 Y	9.02/1.8
b	0.716	0.345	0.353	576.4	19.2	2.6 Y	8.66/2.3
c	0.548	0.348	0.348	576.6	16.8	2.1 Y	7.75/2.0

measure of luminous reflectance, and $(A - B)/G$, called "yellowness," which essentially measures the slope of the spectral reflectance curve away from neutral toward the yellow.

An application of this instrument is illustrated in the study of color change in dehydrated carrots with storage at different temperatures. Typical results are given in Table IV. The measurements were made on the dry material packed level in a tray designed to fit at a specific level in the instrument. The instrument is mounted so that the tray rests horizontally and no cover glass is then necessary to hold the sample in place.

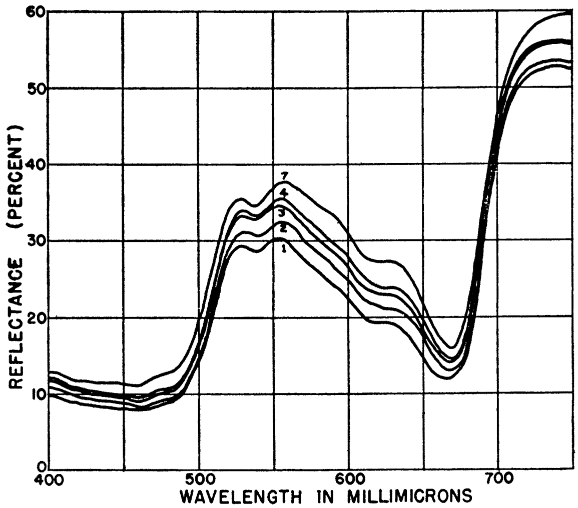


Figure 5. Effect of Delay (at 70° F.) on Reflectance of Peas

(See data in Table III)

Such data give comprehensible information concerning the appearance of the material. It is apparent that temperature is effective in decreasing the natural carrot color. (In this particular instance the yellowness index could perhaps be more aptly labeled "redness," because the typical orange-red carrot color becomes more yellow as the $(A - B)/G$ factor decreases.) It is important to note, however, that the two different methods of treatment result in different color changes. When the G factor is considered, samples treated by process *a* become lighter and less red, while those treated by process *b* become darker and less red as storage temperature increases. Thus process *a* carrots appear bleached, while process *b* carrots are grayed and dull.

The spectral characteristics of the source, photocells, and the three filters are such that approximate I.C.I. tristimulus values may be calculated (*5*) and from these a specification in terms of luminous reflectance, dominant wave length, and purity can be obtained. Hardy has cautioned (*3*), however, that the usefulness of such an instrument as a tristimulus colorimeter depends upon the standardization and constancy of the spectral characteristics of the light source, cell, and filters.

Conversion of Psychophysical Notation into Colorimetrically More Descriptive Terms

The methods described make possible the objective measurement of color of foods and a designation in standardized psychophysical terms. However, the psychological significance of food colors is not directly apparent from results expressed

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch002

Table III. Color Changes in Peas as a Function of Delay before Cooking

(Data obtained from reflectance spectrophotometric curves shown in Figure 5)

Days Held before Cooking	Y	x	y	λ, mμ	p, %	Munsell Notation Hue	Munsell Notation Value/chroma
1	0.254	0.360	0.442	565.1	47.2	5.1 GY	5.58/5.6
2	0.270	0.366	0.437	567.7	47.6	3.8 GY	5.73/5.4
3	0.292	0.367	0.438	567.8	48.0	3.7 GY	5.93/5.6
4	0.296	0.365	0.436	567.6	47.0	3.8 GY	5.96/5.6
7	0.319	0.368	0.428	569.0	45.8	2.8 GY	6.15/5.2

in I.C.I. notation. It is difficult, if not impossible, to visualize the color specified by values of luminous reflectance and chromaticity (Y, x, y) or even by values of dominant wave length and purity. Furthermore, even if the instrumental measurements result in somewhat different values of luminous reflectance and chromaticity, care must be exercised in interpreting these differences in terms of differences apparent to the observer. Equal distances in the I.C.I. chromaticity diagram do not mean equal visual differences.

Conversion tables and charts now available make it possible to express I.C.I. data in forms in which a specified color and the significance of measured color differences can be more easily visualized. For example, I.C.I. values calculated from objective instrumental readings can be converted into the Munsell notation which evaluates the three psychological color attributes—hue, lightness (Munsell value), saturation (Munsell chroma)—on scales of approximately equal visual steps. In addition, the Munsell color charts offer one of the most convenient sources of material standards for direct color comparisons.

Although differences are observed in the I.C.I. data given in some of the illustrative examples above, the psychological significance of these differences is not clear. For instance, there are observed increases in luminous reflectance (Y) and dominant wave length (λ) in the peas with delay before processing (Table III); however, the comparative importance of these two is not clear even though, percentagewise, the Y value increases more than λ. The significance of these differences becomes clearer if conversion is made to Munsell notation. The notations included in Tables II and III were obtained from the I.C.I. specification (Y, x, y) by the method recommended by the Optical Society of America Subcommittee on Spacing of Munsell Colors (*10*). Under ordinary conditions for visual color matching the relation of the steps in the Munsell hue, value, and chroma scales is about as follows: 1 value step = 2 chroma steps = 3 hue steps (for colors of 5 value–5 chroma) (*11, 13*). With this relationship between the scales in mind, it will be noted from the Munsell notations that the peas become lighter (value change = 0.57 unit) and yellower (hue change = 2.3 units) to about the same visually detectable degree. The change in saturation (maximum chroma change = 0.4 unit) is relatively less noticeable. The greater apparent color difference with larger particle size in the potatoes (Table II) is similarly more obvious in the Munsell data than in the I.C.I. data.

If results of color measurements are expressed in Munsell notation, a reader can use Munsell color charts as an aid in visualizing approximate ranges of color differences involved. Such a means has been suggested (*15*) for expressing color of light-colored juices. The necessary experimental data were obtained with a reflection meter similar to the reflectometer described.

Table IV. Effects of Storage Temperature on Color of Dehydrated Carrots

	Yellowness, $(A - B)/G$ [a]	Yellowness, $(A - B)/G$ [b]	Luminous Reflectance, G, % [a]	Luminous Reflectance, G, % [b]
Original sample	1.33	1.27	16.7	17.3
Stored 3 months in N_2 at −30° F.	1.34	1.29	17.3	17.8
Stored 3 months in air at 40° F.	1.31	1.29	17.2	16.4
Stored 3 months in air at 70° F.	1.26	1.21	18.4	15.6
Stored 3 months in air at 100° F.	1.20	1.19	20.3	14.2

[a] Treated with starch.
[b] Treated with ascorbic acid.

The Munsell book standards corresponding to the limiting colors may even serve as material standards for industrial color control. In a material standard system the sample is compared with a standard by eye without the use of any meter or optical instrument. The success and popularity of these systems are largely due to their simplicity of application. The ability of the human eye to compensate for various illuminants and surroundings makes it possible for this system to give results even under mediocre conditions. The most critical work with material standards requires carefully controlled observing conditions.

With the best observing conditions, it is possible for the trained observer to compete with photoelectric colorimeters for detection of small color differences in samples which can be observed simultaneously. However, the human observer cannot ordinarily make accurate color comparisons over a period of time if memory of sample color is involved. This factor and others, such as variability among observers and color blindness, make it important to control or eliminate the subjective factor in color grading. In this respect, objective methods, which make use of instruments such as spectrophotometers or carefully calibrated colorimeters with conditions of observation carefully standardized, provide the most reliable means of obtaining precise color measurements.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch002

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Determination of Amino Acids

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Gravimetric, photometric, chromatographic, enzymatic, and microbiological methods for the determination of amino acids are reviewed and discussed. Marked advances have been made during the present decade in methods applicable to the determination of amino acids, and with the development of new analytical methods it should soon be possible to determine all the amino acids of biological importance with a degree of accuracy sufficient for practical as well as many theoretical purposes.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch003

The attainment of dependable and complete data on the amino acids in plants and animals, proteins and foods, viruses and enzymes, toxins and hormones, and other biological materials is an important objective of current biochemical research. Investigations toward this end were first initiated in 1806 by Vauquelin and Robiquet (*284*), who isolated asparagine from the juice of asparagus shoots. By 1820 the isolation of cystine from a urinary calculus, glycine from gelatin, and leucine from muscle had been reported. Although, as shown in Table I, only nine additional amino acids were identified as products of protein hydrolysis during the ensuing 80 years, fourteen other amino acids have been isolated from plant and animal sources since 1900. [Vickery and Schmidt (*290*) have reviewed the history of the amino acids. Vickery (*285*) has listed amino acids with limited distribution or unsubstantiated claims. β-Hydroxyglutamic acid and norleucine, respectively, were excluded from acceptance because of evidence reported by Dakin (*60*) and Consden *et al.* (*55*).]

Knowledge of the amino acids developed slowly during the 19th century, since Mulder (*200*) and other pioneer workers devoted most of their efforts to the solution of other problems, particularly the elementary composition of proteins. As recently as 1890, Osborne (*211*) determined the elementary composition of oat-kernel proteins in the first of his now-classical investigations on vegetable proteins.

The attention of early workers was directed, also, to the determination of amides in proteins. Amide nitrogen has been determined in many plant and animal products following the report of Nasse (*203*) in 1872 that, during hydrolysis of proteins, a considerable part of the nitrogen was liberated as ammonia. The isolation of glutamine from beetroot juice by Schulze and Bosshard (*243*) in 1883 gave further impetus to these studies. In 1906 Osborne and co-workers (*212*, *215*) found, as shown in Table II, approximate equivalence between the ammonia liberated from plant proteins and that required to form the monoamides from the calculated amounts of aspartic and glutamic acids. It has been concluded more recently, however, from the extensive data on the amide nitrogen and the amides of various plant proteins which have been obtained by Chibnall (*45*, *46*), Vickery, and other workers, that only part of the glutamic and aspartic acids exists in proteins as amides. Chibnall (*47*) and Archibald (*6*, *7*) have reviewed this topic.

A more complete characterization of proteins was proposed in 1899 by Hausmann (*130*), who determined the distribution of nitrogen among amides, the basic amino acids,

In ANALYTICAL METHODS IN THE FOOD INDUSTRY;
Advances in Chemistry; American Chemical Society: Washington, DC, 1950.

Table I. Amino Acids Isolated from Plant and Animal Products

1806–1820[a]	1820–1900[a]	1900–1949	
Aspartic acid (as amide)	Alanine	β-Alanine	Thiolhistidine
Cystine	Arginine	Canavanine	Threonine[a]
Glycine	Diiodotyrosine	Citrulline	Thyroxine[a]
Leucine	Glutamic acid	Dihydroxyphenylalanine	Tryptophan[a]
	Histidine	Djenkolic acid	Valine[a]
	Lysine	Hydroxyproline[a]	
	Phenylalanine	Isoleucine[a]	
	Serine	Methionine[a]	
	Tyrosine	Proline[a]	

[a] Amino acids isolated from protein hydrolyzates.

and the nonbasic amino acids. In the following decade, Hausmann's method was extended by Osborne *et al.* (*214*, *215*), who determined the nitrogen of the humin, and by Van Slyke (*282*), who estimated the nitrogen of four amino acids.

Table II. Amide Nitrogen of Proteins (*212*)

Protein	Ammonia, % Calculated	Found	Difference
Edestin	2.19	2.28	−0.09
Excelsin	1.99	1.80	0.19
Amandin	3.36	3.70	−0.34
Legumin (vetch)	2.27	2.16	0.11
Phaseolin	2.35	2.06	0.29
Glutenin	2.83	4.01	−1.18
Gliadin	4.39	5.11	−0.72
Zein	2.29	3.61	−1.32
Casein	1.38	1.61	−0.23

The importance of Heinrich Ritthausen's fundamental studies, 1862 to 1899, on analytical procedures for the determination of amino acids in proteins has been emphasized in the biographical sketches which have been presented by Osborne (*210*), Vickery (*289*), and Chibnall (*47*). It is of particular interest to note here the prediction made by Ritthausen about 1870 that the amino acid composition would prove to be the most adequate basis for the characterization of proteins. Ritthausen and Kreusler (*230*) were the first, in 1871, to determine amino acids derived from proteins, and some of the values which they found for aspartic and glutamic acids are given in Table III (cited by Chibnall, *47*, and Vickery, *286*).

Gravimetric Methods

In succeeding years amino acids have been determined largely by gravimetric methods of the type employed by Ritthausen. Old methods have been modified and new ones proposed by investigators interested in improving the procedures and the quality of the data. Recalcitrant amino acid mixtures have been separated, new types of potentially valuable amino acid salts have been prepared, factors to correct for solubility losses have been established, and amino acids have been brought to high purity. More specifically, solubility corrections for silver arginate, histidine nitranilate, lysine picrate, and other salts (*121*, *173*, *243*, *271*, *272*, *276*, *283*, *288*) have been applied to the determination of the basic amino acids by the Kossel (*156–163*, *287*) method. Other amino acid salts whose solubilities have been investigated similarly include proline rhodanilate (*17*), hydroxyproline reineckate (*17*), glycine trioxalatochromiate (*18*), alanine dioxypyridate (*18*), calcium glutamate (*13*), and calcium aspartate (*13*). Crude tyrosine has been purified by extracting tyrosine with glacial acetic acid (*123*), precipitating tyrosine as its ethyl ester hydrochloride (*222*) or its mercuric chloride complex (*128*), adsorbing tyrosine on a carbon col-

Table III. First Analysis of Proteins (*230*)

Protein	Aspartic Acid, %	Glutamic Acid, %
Mucedin (wheat gliadin)	...	25
Maisfibrin (maize glutelin)	1.4	10.0
Gluten-casein (wheat glutelin)	0.33	5.3
Conglutin (lupine)	2.00	3.5
Legumin (broad bean)	3.50	1.5

umn (*293*), and removing cystine as its phosphotungstate (*222*). Leucine and isoleucine have been separated from valine as their lead salts (*175*), valine and alanine have been separated by precipitating the latter as its phosphotungstate (*175*), and leucine has been separated from isoleucine and valine as its methanol-insoluble copper salt (*79*) or its 2-naphthalene sulfonate (*20*).

Some amino acids have been determined satisfactorily by gravimetric methods. In 1908 casein was found to contain 3.81% of arginine in Osborne's (*213, 215*) laboratory and, more recently, values ranging from 3.6 to 3.9% have been obtained by investigators who determined arginine by a gravimetric method as its flavianate (*14, 287*), by photometric analysis (*15*), and by microbiological assay (*120, 134, 136, 138, 184, 265*). Although Hlasiwetz and Habermann (*135*) reported in 1873 that casein contained 29% of glutamic acid, Foreman (*92*) stated in 1914 that most workers had obtained only about 11% of this amino acid. At the same time Foreman isolated 21.8% of glutamic acid from casein after separating glutamic and aspartic acids as their ethyl alcohol–insoluble calcium salts. In 1943 Bailey *et al.* (*13*) found 22.0% by an improved Foreman procedure, and approximately the same value was obtained subsequently by other workers who employed gravimetric (*59, 192, 304*), microbiological (*70, 125*), and other procedures (*180*).

On the other hand, the gravimetric values obtained for some amino acids have not been highly accurate. Citing tyrosine as an example, Osborne and Guest (*213*) concluded in 1911 that 4.5%, the value found by Abderhalden and Voegtlin (*1*) in 1907, was the most dependable of any reported following the isolation of this amino acid from casein by Liebig (*178*) in 1846. It seems probable, however, from recent determinations by photometric methods (*14, 90, 304*) that the true value is 5.5% or higher.

Photometric Methods

Photometric methods were first adapted to the determination of amino acids in 1912. Folin and Denis (*90*) determined tyrosine by means of the blue-colored product formed with phos hotungstic acid, while Fasal (*83*) determined tryptophan colorimetrically as its violet-colored glyoxylic acid complex. As indicated in Table IV, photometric procedures have been proposed for the determination of all the common amino acids. Many types of photometric methods have been described and some procedures have yielded reliable data. [Block and Bolling (*26*) and Mitchell and Hamilton (*193*) have reviewed this topic.] The outstanding photometric methods in this category are those applied to the determination of arginine by Sakaguchi (*235*), methionine by McCarthy and Sullivan (*181*), phenylalanine by Kapeller-Adler (*143*), and tyrosine by Folin and Looney (*91*). Many proteins and biological materials have been analyzed for tryptophan by the original or modified glyoxylic acid method of Shaw and McFarlane (*247*) and the *p*-dimethylaminobenzaldehyde procedure of May and Rose (*191*), but it is probable that many of the values were not highly accurate. [Carpenter (*40*) and Spies and Chambers (*258*) have reviewed photometric methods for the determination of tryptophan.] Factors which have tended

Table IV. Photometric Methods First Used to Determine Amino Acids

Date	Amino Acid	Reagent	Author
1912	Tyrosine	Phosphotungstic acid	Folin and Denis (*90*)
1912	Tryptophan	Glyoxylic acid	Fasal (*83*)
1913	Histidine	*p*-Diazobenzenesulfonic acid	Weiss and Ssobolew (*295*)
1922	Cystine	(Cysteine) phosphotungstic acid	Folin and Looney (*91*)
1925	Arginine	1-Naphthol	Sakaguchi (*235*)
1930	Glycine	*o*-Phthaldialdehyde	Zimmermann (*314*)
1932	Phenylalanine	(*o*- and *p*-nitrobenzoic acid) NH_2OH	Kapeller-Adler (*143*)
1933	Hydroxyproline	(Pyrrol) *p*-dimethylaminobenzaldehyde	Lang (*172*)
1938	Alanine	(CH_3CHO) piperazine–sodium nitroprusside	Fromageot and Heitz (*95*)
1939	Threonine	(CH_3CHO) *p*-hydroxydiphenyl	Block and Bolling (*28*)
1939	Aspartic acid	(Dibromoxalacetic acid) dinitrophenylhydrazine	Arhimo (*10*)
1939	Proline	(Pyrrol) *p*-dimethylaminobenzaldehyde	Guest (*119*)
1940	Leucine	(Acetone) salicylaldehyde	Block *et al.* (*26, 29*)
1940	Isoleucine	(Methyl ethyl ketone) salicylaldehyde	Block *et al.* (*26, 29*)
1940	Valine	(Acetone) salicylaldehyde	Block *et al.* (*26, 29*)
1941	Methionine	Sodium nitroprusside	McCarthy and Sullivan (*181*)
1942	Serine	(HCHO) 1,8 - dihydroxynaphthalene - 3,5 - disulfonic acid	Boyd and Logan (*31*)
1946	Glutamic acid	(β-Formylpropionic acid) 2,4-dinitrophenylhydrazine	Prescott and Waelsch (*226*)
1946	Lysine	(Bromolysine) phosphotungstic-phosphomolybdic acids	Nelson *et al.* (*204*)

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch003

to vitiate the analytical results include side reactions of tryptophan with acids, alkalies, and cystine and the simultaneous formation of colored products with tryptamine, skatole, and other interfering substances. Similarly, many of the data obtained for cystine by the phosphotungstic acid procedure of Folin and Looney (*91*) and the 1,2-naphthoquinone-4-sulfonic acid method of Sullivan (*267, 268*) were not highly accurate, owing to destruction of cystine during alkaline hydrolysis of proteins and other factors.

Unique methods based on new principles have been developed within the past 10 years. Threonine (*27, 28, 249*) is oxidized by lead tetraacetate or periodic acid to acetaldehyde, which is determined by photometric analysis of its *p*-hydroxydiphenyl complex or iodometric titration of its combined bisulfite. Serine is oxidized similarly to formaldehyde, which is determined gravimetrically (*207*) as its dimedon (5,5-dimethyldihydroresorcinol) derivative or photometric analysis (*31*) of the complex formed with Eegriwe's reagent (1,8-dihydroxynaphthalene-3,5-disulfonic acid). It appears that the data obtained for threonine and serine in various proteins by these oxidation procedures are reasonably accurate. [Block and Bolling (*26*) have given data on the threonine and serine content of various proteins.]

Solubility Product

The use of aromatic sulfonic acids as specific precipitants for amino acids was first suggested in 1924 by Kossel and Gross (*158*), who observed that flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) forms slightly soluble salts with the basic amino acids. [Stein *et al.* (*198, 260*) have reviewed this topic.] Subsequently, the behavior of more than 100 aromatic sulfonic acids with as many as 20 amino acids was investigated by Bergmann and his collaborators. Although Bergmann *et al.* (*17*) employed aromatic sulfonic acids as specific precipitants in determining glycine, proline, hydroxyproline, and other amino acids in protein hydrolyzates, data of relatively high accuracy have been obtained largely by methods based on the solubility product principle.

As may be noted in Equation 1,

$$(R' - X_a)(Y - Y_a) = (R^2 - X_b)(Y - Y_b) \tag{1}$$

where R', R^2 = moles of reagent added, X_a, X_b = moles of reagent precipitated, Y_a, Y_b = moles of amino acid salt isolated, and Y = moles of amino acid present, the moles of an amino acid present in solution can be calculated from the moles of reagent added and precipitated, the moles of amino acid salt isolated, and the equilibrium equation relating these quantities. The solubility product method has a sound theoretical basis and it has been applied to the determination of alanine, arginine, glycine, leucine, proline, and other amino acids (*19, 20, 33, 141, 198, 260*). Factors which have tended to limit the use of the solubility product method include the unavailability of suitable reagents, its inapplicability to the basic amino acids, the inconstancy of the experimentally determined solubility product values, and the extremely high precision required in the manipulations.

Isotope Dilution

The isotope dilution principle, first employed by Hevesy and Hobbie (*133*) in 1932 for the determination of lead in ores, was applied by Schoenheimer *et al.* (*241*) to the determination of amino acids. [Shemin and Foster (*248*) have reviewed this topic.] An N^{15}-amino acid derivative was added to a protein hydrolyzate, a sample of the amino acid to be determined was isolated and purified, the excess N^{15} in this product was estimated with the mass spectrograph, and the grams of amino acid originally present were calculated from Equation 2.

$$B = \frac{C_o}{C} - 1A \tag{2}$$

where B = grams of amino acid present, A = grams of isotopic amino acid added, C_o = grams of excess isotopic atom in added amino acid, and C = grams of excess isotopic atom in isolated amino acid. Distinct advantages of this procedure are that the specificity of the precipitant and the degree of solubility of the amino acid derivative are not of critical

importance, because it is not necessary to isolate the amino acid in quantitative yield.

A radioactive-isotope dilution procedure of higher sensitivity than the conventional method has been described recently by Keston *et al.* (*146*). The amino acids in the mixture are converted quantitatively to their I^{131} *p*-iodophenylsulfonates, a large excess of the unlabeled amino acid derivative is added as carrier, and the amino acid derivative is isolated and purified to constant concentration of radioactive isotope. Procedures for the separation of the amino acid derivatives by a countercurrent distribution process, ion-exchange resins (*145*), and paper-partition chromatography (*148*) have been utilized by these investigators. [Craig (*57*), Craig *et al.* (*58*), and Bush and Densen (*37*) have reviewed this topic.] Alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, lysine, proline, and tyrosine have been determined in various proteins by the isotope technique which "allows the estimation of amino acids in protein hydrolyzates with an error which can be estimated to be within 1 to 2%" (*146*, *248*). It is evident from Table V that the amino acid data reported in a recent paper by Keston *et al.* (*146*) are in reasonable agreement with the literature values.

Table V. Percentages of Amino Acids in Proteins (*146*)

	β-Lactoglobulin		Human Hemoglobin		Aldolase	
Amino Acid	Authors	Literature	Authors	Literature	Authors	Literature
Alanine	7.00	6.64	9.82	9.9	8.45	7.87
Glycine	1.54	1.5, 1.4	4.49	...	5.55	6.12
Proline	4.88	4.1, 5.4	4.92	...	5.69	...

Chromatographic Methods

Chromatographic methods, first utilized by Tswett in 1906 in separating the pigments of green leaves, have been employed for the separation of amino acids. [Reviews of the principles and applications of chromatography have been given in recent papers (*4*, *24*, *34*, *38*, *39*, *41–44*, *48*, *49*, *54*, *66–68*, *80*, *94*, *111*, *112*, *129*, *131*, *176*, *185*, *186*, *188*, *201*, *216*, *245*, *261*, *266*, *273*, *274*, *277–280*, *292–294*, *299*, *300*, *302*, *303*, *305*, *312*). A biography of Mikhail Tswett (1872–1920) has been written by Zechmeister (*313*).] For present purposes ion exchange, adsorption, and partition are regarded as chromatographic procedures. Materials which have been used as the stationary phases include zeolites, aluminum oxide, silica, starch, carbon, synthetic resins, and paper. Chromatographic procedures for the separation of amino acids by partition were first proposed by Martin and Synge (*189*) in 1941. The acetylated derivatives of the amino acids were distributed between two partially miscible solvents, such as chloroform and water, in a column of precipitated silica. In 1944 Consden *et al.* (*52*) first separated "free" amino acids by paper-partition chromatography. [The name "papyrography" was suggested by Dent (*62*).] Water-saturated phenol was employed as the moving phase and the amino acids were revealed by the colored spots formed with ninhydrin. Chromatographic methods have been applied extensively to the separation and qualitative identification by means of the R_f values (ratio of the distance traveled by the amino acid to that traveled by the solvent) of many amino acids (*62*), and peptides (*53*, *56*, *174*) in urine (*61–64*, *126*, *275*, *311*), animal tissues (*2*, *3*, *86*, *231*, *298*), tobacco mosaic virus (*153*), Gramicidin S (*56*), bacterial hydrolyzates (*223*), plant cells (*65*, *167*, *179*, *263*), and other biological materials (*11*, *87*, *227*, *306*). [Summaries of the R_f values of amino acids have been given by Dent (*62*) and Martin (*185*).]

Chromatographic methods were first employed for the quantitative determination of amino acids by Martin and Synge (*189*) in 1941. The amino acids were acetylated, their acetyl derivatives were partitioned between two immiscible solvents on precipitated silica, and the colored bands formed with methyl orange were collected and titrated. This method has been applied to the determination of amino acids in the hydrolyzates of wool (*113*, *190*), gelatin (*113*, *115*), gramicidin (*114*, *269*), and other biological materials (*21*, *116*, *117*). Analogous procedures were proposed by Wieland and Fremerey (*301*), who determined amino acids in chromatograms by iodometric titration of their copper salts and by Karrer *et al.* (*144*), who separated amino acids as their *N*-*p*-phenylazobenzoyl derivatives on a basic zinc carbonate column. Procedures have also been suggested for the

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch003

quantitative separation of amino acids as their *N*-2,4-dinitrophenyl derivatives (*236*) and their *N*-azobenzene *p*-sulfonyl derivatives (*229*).

A limitation of chromatographic methods for the quantitative determination of amino acids has been the necessity of employing accessory methods for the analysis of the chromatograms. Cannan (*39*) and Kibrick (*149*) determined aspartic and glutamic acids in protein hydrolyzates by electrometric titration and ninhydrin analysis of chromatograms prepared by means of the polyamineformaldehyde resin, Amberlite IR4. Pratt and Auclair (*225*) have investigated the sensitivity of the ninhydrinamino acid reaction and Moore and Stein (*197*) the color yields. Similarly, amino acids in chromatograms have been determined in terms of Kjeldahl nitrogen (*240*) and amino nitrogen (*278–280*). Bergdoll and Doty (*15*) analyzed chromatograms for lysine by the ninhydrin method, histidine by Pauly's diazo procedure, and arginine by the Sakaguchi reaction. Amino acids in chromatograms developed on paper have been determined by photometric analysis of the ninhydrin (*63*, *202*, *224*, *225*, *261*) or the 2-naphthoquinone sulfonate (*12*) colored complex as well as in terms of the areas of ninhydrin spots (*89*) and the areas under curves obtained by plotting color densities against the distances of ninhydrin spots from the starting line (*25*) or by plotting percentage light transmittance through ninhydrin chromatograms against the distances along the paper strips (*35*). Related methods which have been suggested include determination of the optical density of the yellow product formed by the reaction of copper complexes of the amino acids with sodium diethyl dithiocarbamate (*307*, *308*), polarographic response of copper complexes of the amino acids (*187*), and radioactivity of I^{131} *p*-iodobenzene sulfonyl derivatives of amino acids (*147*, *148*).

Two chromatographic methods reported recently for the quantitative determination of amino acids are of particular interest. Stein and Moore (*196*, *261*) have described a procedure for the quantitative chromatographic separation of six amino acids on a starch column with a solvent consisting of 1-butanol, benzyl alcohol, and water. Effluent fractions were collected with the aid of an automatic fraction-collecting machine, the amino acids in the effluent fractions were determined by photometric ninhydrin analysis, effluent concentration curves were constructed, and the resulting peaks were integrated to give the amino acid concentrations in the fractions. Mixtures of amino acids with 19 components corresponding in composition to protein hydrolyzates were analyzed for a number of amino acids with a limiting accuracy of $\pm 3\%$. The percentages of six amino acids in β-lactoglobulin and bovine serum albumin determined by this chromatographic-ninhydrin procedure are shown in Table VI. Most of the values were in good agreement with those reported in the literature.

Table VI. Percentages of Amino Acids in Proteins

Amino Acid	β-Lactoglobulin			Bovine Serum Albumin	
	PCS	MBA	Other methods	PCS	Other methods
Isoleucine	5.86	6.1–8.7	...	2.61	2.9 MBA
Leucine	15.5	15.3	15.7 ID 15.9 SP	12.3	13.7 MBA
Methionine	...	...	...	0.92	0.81 ID
Phenylalanine	3.78	3.5, 4.3	4.2 CSG	6.60	6.2 MBA
Tyrosine	3.64	...	3.8 Phot.	5.06	5.49 Phot. 5.53 ID
Valine	5.62	5.5, 5.8	5.8	5.22	6.5 MBA

PCS. Partition chromatography on starch column (*196*, *261*).
MBA. Microbiological assay.
ID. Isotope dilution.
CSG. Chromatography on silica gel column.
SP. Solubility product.
Phot. Photometric.

The percentages of amino acids in silk fibroin which Polson *et al.* (*224*) found by direct visual and indirect photometric analysis of ninhydrin paper-partition chromatograms are shown in Table VII. The percentages obtained for alanine, glycine, and serine appear to be reasonably accurate, inasmuch as they agree closely with those found by other methods. It would be of interest to determine alanine by the microbiological method reported recently by Sauberlich and Baumann (*238*), in view of the widely different values found for this amino acid by the described ninhydrin-chromatographic procedure and the selec-

tive precipitation method of Bergmann and Niemann (*65*). Although the amino acids present in low concentration were not detected, the procedure of Polson *et al.* is rapid, convenient, and particularly applicable to amino acids which are present in relatively high concentrations.

Table VII. Percentages of Amino Acids in Silk Fibroin

Amino Acid	Indirect Color Analysis[a]	Direct Color Analysis		Literature	
		Visual[a]	Photometric[b]	Value	Method
Alanine	37.6	34.0	35.2	26.4	Sp-1
Arginine	2.4	0.8	...	0.76	Sp-2
Glutamic acid	...	...	...	2.03	MBA
Glycine	39.9	42.4	42.4	43.8[c]	Sp-1
Histidine	...	...	...	0.34	MBA
Isoleucine	Trace	2.5	...	...	...
Lysine	...	...	...	0.6	MBA
Methionine	Absent	Absent	...	0.14	MBA
Phenylalanine	Absent	Absent	...	1.3	MBA
Proline	Trace	Trace	...	0.57	MBA-1
Serine	12.7	11.9	...	13.6	PO
Threonine	...	...	...	1.2	MBA
Tyrosine	5.9	8.3	...	13.2	SP-1
Valine	4.4	5.7	...		

[a] Polson *et al.* (*224*).
[b] Dunn and Rockland (*75*).
[c] 43.6 (MBA) (*246*).
Sp-1. Bergmann and Niemann (*18*).
Sp-2. Vickery (*287*).
PO. Nicolet and Shinn (*208*).
MBA. Dunn *et al.* (*70–74, 76, 77, 246*).

Enzymatic Methods

Amino acids were first determined quantitatively by enzymatic methods by Jansen (*142*) in 1917. [Archibald (*5*) has reviewed this topic.] Arginine was split by arginase into ornithine and urea and the urea was converted to ammonium carbonate with urease. These enzymatic procedures were later improved by Hunter and Dauphinee (*139*) and they have been utilized (*139, 287*) to determine arginine in various proteins. In 1937 Virtanen and Laine (*291*) determined lysine by estimation of the cadaverine formed on decarboxylation of this amino acid with *Bacillus coli.* Basic studies leading to quantitative methods for the determination of L-amino acids with L-amino acid decarboxylases derived from bacteria were initiated by Gale (*99*) in 1940. [Gale (*97, 98*), Blaschko (*22*), and Werle (*297*) have reviewed this topic.] As shown in Table VIII, six amino acid decarboxylases have been prepared from the indicated bacterial strains. As shown in Table IX, nine to thirteen of the fourteen strains of coliform organisms investigated exhibited decarboxylase activity for each of five amino acids. The specificity of the bacterial decarboxylases was indicated by their distribution among the strains of organisms. Since that date the decarboxylases have been extensively investigated by Gale and co-workers (*81, 82, 98, 100–109, 270*). That the L-lysine decarboxylase of *Bacterium cadaveris* 6578 might be adapted to the quantitative determination of this amino acid was suggested by Gale and Epps (*109*) in 1943. The next year Neuberger and Sanger (*205, 206*) and Zittle and Eldred (*315*) described L-lysine decarboxylase procedures which were applied to the determination of L-lysine in various proteins. As indicated in Table X, Gale (*103, 106*) determined six amino acids in a series of proteins by decarboxylase methods. In all but a few cases—indicated as underlined values in the table—the percentages of amino acids found were in close agreement with the literature data. It has been reported recently by Hanke (*127*) that the decarboxylation of L-lysine and L-tyrosine yields nearly the theoretical carbon dioxide when oxygen is eliminated or L-leucine is added to solutions of these amino acids. Procedures for the determination of glutamic acid and glutamine by estimation of ammonia and the carbon dioxide liberated by the action of decarboxylases obtained from *Clostridium welchii* SR12 have been described recently by Krebs (*164*). Archibald (*5–9*) has described an enzymatic procedure for the determination of glutamine with glutaminase obtained from kidney. Blaschko and Stanley (*23*) have prepared a tyrosine decarboxylase from *S. faecalis* which decarboxylates other aromatic amino acids with a para phenolic group, such as 3,4-dihydroxyphenylalanine (Dopa), and a Dopa decarboxylase from mammalian liver with decarboxylation activity limited to aromatic amino

acids with meta phenolic groups. An L-glutamic acid decarboxylase which Schales *et al.* (*239, 240*) isolated from squash has been applied to the determination of L-glutamic acid in various proteins.

Table VIII. Amino Acids Determined by Decarboxylase Methods

(Bacterial sources of decarboxylases, *104*)

Amino Acid	Organism	pH	Temp., ° C.
Arginine	*E. coli* (7070)[a]	5.2	25
Glutamic acid	*Cl. welchii* SR12 (6784)	4.5	37
Histidine	*Cl. welchii* BW21 (6785)	4.5	37
Lysine	*B. cadaveris* (6578)	6.0	25
Ornithine	*Cl. septicum* PIII (547)	5.5	37
Tyrosine	*S. faecalis* (6783)	5.5	37

[a] National Type Culture Collection (London) number.

Microbiological Methods

Microbiological methods for the quantitative determination of amino acids was first reported by Kuiken *et al.* (*165*) less than 6 years ago. The procedures utilized by these investigators were essentially the same as those first employed by Snell and Strong (*256*) in 1939 to determine the vitamin, riboflavin. It is recognized that all microbiological assay procedures in common use today have resulted from the countless experiments of early workers from the time of Pasteur (*217, 218*), who studied the growth and metabolism and determined the nutritional requirements of yeasts, pathogenic bacteria, and other organisms on basal media containing chemically defined components.

Table IX. Amino Acid Decarboxylases in Coliform Organisms (*99*)

Strain	Ornithine	Arginine	Lysine	Histidine	Glutamic Acid
Bact. coli 217	+	+	+	+	+
Bact. coli Esch.	+	+	+	+	−
Bact. coli 210	−	+	+	−	+
Bact. coli 201	−	−	+	+	+
Bact. friedländeri	+	−	+	+	−
Bact. coli faecal B	+	−	−	−	−

The nutrition of lactic acid bacteria has been reviewed by Burrows (*36*), Clifton (*50*), Henneberg (*132, 177*), Kluyver (*150*), Knight (*151, 152*), McIlwain (*182*), Koser and Saunders (*155*), Orla-Jensen (*209*), Peskett (*220*), Peterson and Peterson (*221*), Snell (*253–255*), Stephenson (*262*), and Werkman and Wood (*296*). Microbiological procedures for the quantitative determination of amino acids have been reviewed by Snell (*244, 252, 253*) and Dunn (*69*). Microorganisms other than lactic acid bacteria utilized to determine amino acids include *Clostridium perfringens* BP6K (13 amino acids) (*32*), *E. coli* 1577–28 (arginine) (*168, 170*), *Tetrahymena geleii* H (histidine) (*232*), *Tetrahymena geleii* H (tryptophan) (*242*), *Neurospora crassa* 33757 (leucine) (*137, 228, 234*), *E. coli* 679–680 (leucine) (*251*), *E. coli* 522–171 (*169, 170*), *E. coli* mutant (tryptophan) (*170*), and *E. coli* 58–5030 (*250*).

Some of the most notable contributions were (*a*) the discovery of numerous strains of lactic acid bacteria including those listed in Table XI, (*b*) the elaboration of the mineral

Table X. Percentages of Six Amino Acids in Proteins

(Given as N − % of total nitrogen)

Protein	Arginine		Glutamic Acid[a]		Histidine[b]		Lysine[b]		Ornithine		Tyrosine[c]	
	d	*e*	*d*	*e*	*d*	*e*	*d*	*e*	*d*	*e*	*d*	*e*
Edestin	27.9	28.7	..	..	3.66	4.1	2.44	2.44	..	..	2.61	2.56
Fibrin	..	..	7.98	8.25	4.67	3.95	10.4	11.3	..	..	1.59	1.33
Gliadin	..	..	23.4	25.3	3.53	3.30	0.79	0.7	..	..	1.41	1.43
Hemoglobin	6.92	6.95	4.42	3.76	12.7	12.5	10.9	9.4	..	..	5.05	6.05
Insulin	6.36	6.35	..	..	8.4	8.4	..	..	..	..	..	..
Tyrocidin	..	..	7.06	7.2	..	..	..	..	13.1	13.2	7.4	6.8

[a] Recovery 93% from amino acid test mixture.
[b] Recovery 100.6% from amino acid test mixture.
[c] Recovery 99.2% from amino acid test mixture.
[d] Decarboxylase-CO_2 method (*103, 106*).
[e] Literature method.

requirements of lactic acid and other organisms by Henneberg (*132*), Ushinsky (*281*), Speakman (*257*), and other workers, (*c*) the investigations by Orla-Jensen (*209*) on the morphology, nutrition, and vitamin (pantothenic acid and riboflavin) requirements of lactic acid bacteria, (*d*) the studies by Fred and his collaborators (*93*) on the fermentative products and processes of lactic acid bacteria, and (*e*) the observations of Möller (*195*) that pyridoxine and biotin stimulated the growth of lactic acid bacteria. Other studies of particular significance were those of Koser and Rettger (*154*), Fildes *et al.* (*84, 85*), Mueller (*199*), Gladstone (*110*), McIlwain (*183*), Landy and Dicken (*171*), Peterson *et al.* (*30, 140*), Pelczar and Porter (*219*), Gaines and Stahly (*96*), Werkman *et al.* (*309, 310*), Shankman (*245*), and numerous other investigators of the amino acid nutrition and metabolism of lactic acid and other bacteria.

Table XI. History of Discovery of Common Lactic Acid Bacteria (*16*)

Organism	Discoverer	Date
Streptococcus lactis	Lister	1873
Leuconostoc mesenteroides	Cienkowski, Van Tiegham	1878
Lactobacillus casei	von Freudenreich	1890
Lactobacillus lactis	Leichmann	1896
Lactobacillus delbrueckii	Leichmann, Lafar	1896
Lactobacillus acidophilus	Moro	1900
Lactobacillus fermenti	Beijerinck	1901
Lactobacillus buchneri	Henneberg	1903
Streptococcus faecalis	Thiercelin	1902
Streptococcus salivarius	Andrewes, Horder	1906
Leuconostoc dextranicum	Beijernick	1912
Lactobacillus plantarum	Orla-Jensen	1919
Lactobacillus pentoaceticus	Fred, Peterson, Davenport	1919
Leuconostoc citrovorum	Hammer	1920
Lactobacillus arabinosus	Fred, Peterson, Davenport	1921
Lactobacillus pentosus	Fred, Peterson, Davenport	1921
Lactobacillus lycopersici	Mickle	1924

In more recent times chemically defined basal media have been elaborated, on which the growth of various lactic acid bacteria is luxuriant and acid production is near-optimal. The proportions of the nutrients in the basal media have been determined which induce maximum sensitivity of the organisms for the test substance and minimize the stimulatory or inhibitory action of other nutrilites introduced with the test sample. Assay conditions have been provided which permit the attainment of satisfactory precision and accuracy in the determination of amino acids. Experimental techniques have been provided which facilitate the microbiological determination of amino acids. On the whole, microbiological procedures now available for the determination of all the amino acids except hydroxyproline are convenient, reasonably accurate, and applicable to the assay of purified proteins, food, blood, urine, plant products, and other types of biological materials. On the other hand, it is improbable that any microbiological procedure approaches perfection and it is to be expected that old methods will be improved and new ones proposed by the many investigators interested in this problem.

Table XII. Microbiological Assay Methods First Used to Determine Amino Acids

Date	Amino Acid	Organism	Investigator	Date	Amino Acid	Organism	Investigator
1943	Isoleucine	*L. arab.*[a]	Kuiken *et al.* (*165*)	1945	Histidine	*L. mesen.*	Dunn *et al.* (*72*)
	Leucine	*L. arab.*	Kuiken *et al.* (*165*)		Methionine	*S. faec.*[c]	Stokes *et al.* (*265*)
	Valine	*L. arab.*	Kuiken *et al.* (*165*)		Threonine	*S. faec.*	Stokes *et al.* (*265*)
	Tryptophan	*L. arab.*	Greene and Black (*118*)		Phenylalanine	*L. delbr.*	Stokes *et al.* (*265*)
1944	Arginine	*L. casei*	McMahan and Snell (*184*)	1946	Tyrosine	*L. delbr.*	Gunness *et al.* (*122*)
	Glutamic acid	*L. arab.*	Dunn *et al.* (*70*)		Proline	*L. mesen.*	Sauberlich and Baumann (*237*)
	Lysine	*L. mesen.*[b]	Dunn *et al.* (*72*)		Cystine	*L. mesen.*	Sauberlich and Baumann (*237*)
1945	Aspartic acid	*L. delbr.*[d]	Stokes and Gunness (*264*)	1947	Glycine	*L. mesen.*	Shankman *et al.* (*246*)
	Serine	*L. delbr.*	Stokes and Gunness (*264*)	1949	Alanine	*L. citr.*[e]	Sauberlich and Baumann (*238*)

[a] *Lactobacillus arabinosus* 17-15.
[b] *Leuconostoc mesenteroides* P-60.
[c] *Streptococcus faecalis* R.
[d] *Lactobacillus delbrueckii* LD5.
[e] *Leuconostoc citrovorum* 8081 (American Type Culture Collection number).

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch003

As shown in Table XII, a microbiological assay procedure is available for the determination of each of 18 amino acids. The original methods indicated in the table have been modified and in many instances greatly improved by later workers, although it is not possible here to give any account of the extensive investigations that have been made in this field. There are potentialities for improved methods and assays through better balance of nutrilites in basal media, the use of different strains of assay organisms, and increased precision through the refinement of experimental assay techniques. Dunn *et al.* (*78*) have shown that as many as 15 amino acids are essential for the growth of some strains of lactic acid bacteria.

As is illustrated by the data given in Table XIII, knowledge of the amino acids in proteins and other biological materials has been increasing slowly. The percentages of amino acids in casein given by Foreman (*92*) in 1919 resembled closely those accepted by Osborne and Guest (*213*) in 1911. The most striking differences are the increase in glutamic acid from about 15 to 22% and in glycine from 0 to 0.45%. In 1943 Cohn and Edsall (*51*) listed values for cystine, methionine, and threonine and gave increased percentages of aspartic acid and serine. Since that date values significantly higher than those recorded by Cohn and Edsall have been reported for alanine, aspartic acid, glycine, histidine, phenylalanine, proline, and threonine, as well as a lower percentage for valine. Furthermore, dependable individual percentages for leucine and isoleucine have replaced the nonspecific values found previously for the sum of these amino acids. It has been possible, therefore, to increase the total amino acids found per 100 grams of casein from 64 to 107%, to a large extent, through the availability of microbiological assay procedures.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch003

Table XIII. Percentages of Amino Acids in Casein

Amino Acid	1911, Osborne and Guest (*213*)	1919, Foreman (*92*)	1943, Cohn and Edsall (*51*)	1949 Value	1949 Investigator	1949 Date	1949 Ref. No.	1949 Method[a]
Alanine	1.5	1.85	1.85	3.7	Sauberlich and Baumann	1949	(*238*)	MBA
Arginine	3.81	3.81	3.72	3.8	Horn, Jones, and Blum	1948	(*138*)	MBA
Aspartic acid	1.39	1.77	5.95	7.0	Hac and Snell	1945	(*124*)	MBA
Cystine	...	...	0.42	0.40	Williamson	1944	(*304*)	Phot.
Glutamic acid	15.55	21.77	21.6	22.0	Bailey *et al.*	1943	(*13*)	Grav.
Glycine	0.0	0.45	0.45	1.9	Shankman *et al.*	1947	(*246*)	MBA
Histidine	2.50	2.5	2.50	3.0	Dunn *et al.*	1945	(*73*)	MBA
Hydroxyproline	0.23	0.23	0.23	0.23	Fischer	1903	(*88*)	Grav.
Isoleucine (Isoleucine + Leucine)	9.35	9.70	9.70	5.6	Stokes *et al.*	1945	(*265*)	MBA
Leucine				9.3	Kuiken *et al.*	1943	(*166*)	MBA
Lysine	5.95	7.62	6.25	7.7	Stokes *et al.*	1945	(*265*)	MBA
Methionine	...	...	3.25	3.0	Dunn *et al.*	1946	(*71*)	MBA
Phenylalanine	3.20	3.88	3.88	4.9	Dunn *et al.*	1945	(*76*)	MBA
Proline	6.70	7.63	8.7	10.5	Dunn *et al.*	1949	(*74*)	MBA
Serine	0.50	0.35	5.0	5.0	Nicolet and Shinn	1941	(*207*)	Ox.
Threonine	...	...	3.5	4.3	Dunn *et al.*	1946	(*77*)	MBA
Tryptophan	1.50	1.5	1.54	1.3	Williamson	1944	(*304*)	Phot.
Tyrosine	4.50	4.5	5.36	5.5	Williamson	1944	(*304*)	Phot.
Valine	7.20	7.93	7.93	6.7	McMahan and Snell	1944	(*184*)	MBA
Total	63.88	75.49	91.8	105.8				

[a] MBA, microbiological assay. Phot., photometric. Grav., isolation. Ox., oxidation.

Conclusions

Marked advances have been made during the present decade in methods applicable to the determination of amino acids. As recently as 1941 Vickery (*286*) listed the amino acids in three categories according to the degree of accuracy with which they could be determined. The eight amino acids concerning which information was only qualitative included the four amino acids (isoleucine, serine, threonine, and valine) which are determinable with reasonable accuracy at the present time by microbiological and other methods. The six amino acids for which methods of a considerable degree of probable accuracy had been proposed were alanine, glycine, hydroxyproline, leucine, phenylalanine, and proline. Microbiological and other methods which may be more satisfactory than classical procedures are now in common use for the determination of all these amino acids except hydroxyproline. No method available today is adequate for the quantitative determination of hydroxyproline, although it is probable that this amino acid could be de-

termined satisfactorily by solubility product, isotope dilution, and paper-partition chromatography procedures. There were nine amino acids (arginine, aspartic acid, cystine, glutamic acid, histidine, lysine, methionine, tyrosine, and tryptophan) for which existing methods appeared to give satisfactory results. Of these amino acids, all except three can be determined at the present time by microbiological and other methods with an accuracy which in some instances appears to be somewhat higher than that attainable by the classical methods in vogue in 1941. Although tyrosine can be determined with reasonable accuracy by photometric and microbiological methods, difficulties still persist in the determination of cystine and tryptophan, owing to the decomposition of these amino acids during treatment of proteins and other biological materials with acid or alkali.

Proteins have been hydrolyzed by treatment with sulfuric acid, hydrochloric acid, barium hydroxide, proteolytic enzymes, and other hydrolytic reagents, but no condition has been found which avoids some destruction or incomplete liberation of tryptophan, cystine, and some other amino acids. The early work on this problem has been reviewed by Mitchell and Hamilton (*194*). The literature and their own excellent experiments on the hydrolysis problem in relation to the liberation and destruction of tryptophan have been presented recently by Spies and Chambers (*259*).

The time is approaching when, because of the development of new analytical methods, it should be possible to determine all the amino acids of biological importance with a degree of accuracy sufficient for practical, as well as many theoretical purposes.

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Quality Control Methods in Frozen Food Production and Distribution

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The successful and profitable control of frozen food quality requires workable standards of product quality and condition and suitable methods for determining the degree of product conformance with standards. The frozen fruit and vegetable industry has developed rapidly during the past 20 years and serious concentration upon the development of adequate standards and objective methods for quality measurement is necessary. Such standards and methods should be placed on a uniform basis the country over and given official and legal status.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch004

The term "quality control" has been widely and loosely employed in the frozen food industry. In the dynamic sense it means the application and control of those techniques of raw material selection, handling, processing, warehousing, and distribution which are known to be required for the production and maintenance of a given level of product quality and condition.

In the final analysis, market price and sales volume are functions of the quality standards offered and the buyer's degree of confidence that the product will conform to the standards. Maintenance of buyer's confidence requires inspection to screen out all nonconforming products, or control over variability of quality during production and distribution to a degree where few, if any, products fail to meet the standards. Screening inspection of the finished product cannot improve quality; it merely serves to segregate unacceptable from acceptable product, and results in loss of production capacity and costly waste and salvage. The second consideration provides the only sound basis for quality control in frozen food production and distribution. It operates on the old principle that "an ounce of prevention is worth a pound of cure."

The successful and profitable conduct of a frozen food quality program requires suitable and workable standards of product quality and condition, and suitable methods for determining the degree of product conformance with the standards. The methods used may be either subjective or objective in character, or a combination of both; the most consistent and reproducible results will be obtained with objective methods. Present-day frozen food standards are, for the most part, based upon subjective methods of quality determination. These are certainly better than none at all; but they are subject to considerable misinterpretation and human error, and for this reason leave much to be desired in providing a sound basis for quality control.

An essential step in the program is production-line control for the early detection of deviations from the standard and the initiation of corrective actions. Objective methods are of value here, but this value may be considerably limited unless methods are sufficiently simple and rapid to give results that may be acted upon during production. For this reason they can generally be little more than qualitative or semiqualitative in character. This should not constitute a deterrent to their development and use, so long as

In ANALYTICAL METHODS IN THE FOOD INDUSTRY;
Advances in Chemistry; American Chemical Society: Washington, DC, 1950.

they are based upon correlation with more refined methods and tolerances are set up allowing for a reasonable margin of safety.

For examination of finished product, where time is not a limiting factor, more precise and accurate methods may be used to advantage for checking the reliability of production control methods, for settling claims concerning quality and condition, for tracing the causes of quality deterioration, and for fixing responsibility.

Quality control is usually thought of in terms of control during production, and in some industries this may be all that is required. In the food industry such control must extend into the channels of distribution. The highly perishable nature of frozen foods makes this phase extremely important. It deserves a great deal more emphasis than it has received in the past.

In the interests of presenting a general outline of the problems involved in quality control and measurement, the remainder of this discussion is chiefly concerned with an important and representative product—frozen peas.

Quality Control in Production and Distribution of Frozen Peas

Maturity. With the harvesting of the crop comes the first opportunity for application of objective measures of quality. The stage of maturity at which the crop is harvested is of great importance, for it is capable of having a marked effect upon the color, flavor, and texture of the finished product.

Decision regarding the time for harvesting a field of peas generally rests with the field department or the grower, and is almost wholly dependent upon experience and judgment. This requires that a measurement of maturity be applied as the product is received at the plant. This is generally used as a basis for grower payment. It makes possible segregation of the raw material for diversion into the proper processing line for a given level of quality, or its complete rejection.

Among the chemical methods proposed for the objective measurement of maturity may be listed total solids, alcohol-insoluble solids, and starch, based upon the fact that these constituents increase in concentration with maturity. Each has its limitations and advantages, and all are equally applicable as long as the limitations are taken into account. These methods as they apply to frozen peas have been discussed by Nielsen and co-workers (*31*) and by Lee (*22*).

With the possible exception of the technique described by Nielsen (*30*, *32*) for the rapid estimation of starch, none of these methods is applicable to production-line control because of the time limitations inherent in them.

Other methods, which are more or less applicable to production-line control, have as their basis the measurement of density or specific gravity, which increases in peas with increase in maturity. One of these, which is generally used because it is the basis for the grading of "tenderness and maturity" in the United States standards for grades of frozen peas, is based upon determining the percentage of peas by count which sink in sodium chloride solutions of 13, 15, and 16% concentration, enabling classification into A, B, C, and D grades.

To be effective, this method must be carried out on samples which have been blanched, and upon peas from which the skins have been removed. The heat applied in blanching drives off gases entrapped in the tissues, and removal of the skins is required to remove air that may be entrapped under them, although it materially slows up the operation and makes it very tedious. In order that there may be consistency in grading, the test must be conducted under closely standardized conditions of temperature and solution concentration. This becomes of considerable importance in borderline cases, and failure to take it into consideration no doubt accounts for some of the inconsistency in results experienced by the industry. The test is not a true measure of tenderness, in that it accounts for variation in skin texture only in so far as maturity affects skin texture. Skin texture is affected by factors other than maturity (*4*). Other methods for the estimation of maturity based upon density or specific gravity have been suggested by Jodidi (*15*) and by Lee (*22*).

A method for maturity measurement, which has come into widespread use and is

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch004

particularly adaptable to production-line control, is based upon the measurement of the force required to shear a sample of peas through a standard grid. The instrument used for this purpose is known as the tenderometer (*25*). Martin and co-workers (*26*) and Walls, Kemp, and Stier (*37*) have shown that there is a high degree of correlation between tenderometer values, maturity, and the texture characteristics of canned peas.

Campbell (*6*) was perhaps the first to investigate the relationship between tenderometer values for raw peas and the texture characteristics of collateral samples after freezing and cooking. A high degree of correlation was observed. The investigations of Nielsen and co-workers (*31*) indicate the usefulness of the instrument for estimation of maturity in peas intended for freezing preservation. In order to secure consistent and reproducible results, the instrument requires standardization and calibration, and the measurements must be made under standardized conditions of temperature and interval after harvest.

A smaller, less expensive, but less accurate instrument, known as the texturemeter, has been discussed by Walls, Kemp, and Stier (*37*) and by Lee (*22*). Because of its small size it should be applicable to field use. Kramer (*18*) has indicated that another instrument, which amounts to a miniature tenderometer adaptable to field use, is in the process of development.

The tenderometer is not readily adaptable for measurement of maturity in the finished product, and any method used for estimating maturity at this point should yield results well correlated with those obtained by the tenderometer. Estimation of total solids or starch content appears to fulfill this requirement very well.

Handling of Peas

The elapsed interval between harvesting and processing has a substantial effect upon quality. Delays in handling after harvesting may result in abnormal colors, flavors, loss of sweet taste, and toughening of skins. Because shelled peas as they come from the fields contaminated with pod and vine juice provide an excellent medium for bacterial growth, undue delays result in bacterial spoilage accompanied by the development of a sour odor and by color deterioration. Even without the influence of this factor, deterioration may occur due to the physiological activities of the tissues.

At present there is little, if any, application of objective methods for determination of product condition or freshness prior to commencement of processing. Such methods would be useful, if for no other reason than to convince the production department that the material in question is no longer suitable for meeting a given standard of quality. There is no question when deterioration has advanced to the stage where it is discernible to the sense of sight or smell; at this stage it is obvious that the material is unfit for use. It is the incipient stages of deterioration with which one should be concerned in this case.

Because physiological deterioration is generally accompanied by an increase in bacterial population, as pointed out by Nielsen, Wolford, and Campbell (*33*), estimation of bacterial numbers might serve as the basis of a test for condition. Obviously, the plate count method is not adaptable because of the time limitations imposed. Direct microscopic count would be much more appropriate, especially if a positive field count were substituted for cell count as suggested by Wolford (*39*).

An even simpler and perhaps more effective approach to the problem might be application of the resazurin test as applied in the milk industry for indirect estimation of bacterial population. Proctor and Greenlie (*34*) have suggested this application, and Wolford (*38*) has worked with it in a limited way. The test is based upon a color change involved in the reduction of the dye. The time required for reduction decreases with increase in bacterial numbers. Intensive investigation of this method and its application with respect to increasing its sensitivity, and correlation of reaction rates with bacterial population and quality characteristics of the product, might be of considerable value.

Misleading interpretation might result if bacterial population estimation were applied to material held under refrigerated conditions. Under such conditions bacterial development is materially held in check, while physiological deterioration still continues to

some extent. This relationship is illustrated in the work reported by Nielsen, Wolford, and Campbell (*33*).

Approaching the matter from an entirely different angle, a semiquantitative estimation of ascorbic acid or total iodine-reducing substances might provide a suitable basis. Delays in handling involve rather marked losses of ascorbic acid. Kramer and Mahoney (*20*) have observed a relationship between quality and the amount of iodine-reducible substances remaining in lima beans.

Processing

A basic step in the preparation of peas and other vegetables for freezing preservation is the treatment referred to as "blanching." This treatment might properly be called "scalding," for it involves a short period of heating with hot water or steam, to prevent flavor and color deterioration during subsequent freezing storage. It also brightens the green color and sets it, so that it is more resistant to color change upon subsequent cooking of the product.

There is a relationship between degree of blanching, quality retention during storage, and the degree of enzyme activity to be found in the tissues. Consequently, tests for the degree of enzyme activity, such as catalase and peroxidase, have been applied as criteria of adequate blanching. Qualitative or semiquantitative tests of this kind lend themselves well to production-line control. Because of the rather wide variety of methods available for the determination of catalase or peroxidase activity, there is still a certain amount of confusion concerning their interpretation when applied as criteria for blanching. There is a real need for the standardization of such techniques throughout the industry.

An early application, still in use for the estimation of catalase activity, consists of suspension of macerated blanched tissue in 3% hydrogen peroxide solution. The test is considered negative, and blanching adequate, in the absence of the formation of a constant stream of bubbles arising from the tissue surfaces. In a great many instances if the reaction mixture is allowed to stand sufficiently long, bubbling will be observed in material known to have been adequately blanched. This has been a source of dissatisfaction with the method. However, in the case of peas and corn, any question concerning this delayed action can be eliminated by removing the skins prior to preparation of the tissue for test. Possibly in recognition of the insignificance of this delayed action, Joslyn (*16*) introduced a time limit of 2 minutes for the observation.

Another variation which attempts to place the test upon a semiquantitative basis involves carrying out the reaction in a Smith fermentation tube. This enables one to obtain a rough idea of the volume of gas formed, but it can be misleading unless a time limit is imposed and the skins are removed.

Quantitative tests for catalase activity find their greatest usefulness in examination of finished product. For this purpose gasometric methods (*36*) or chemical methods based upon measurement of residual hydrogen peroxide (*2*) may be used. In the use of these quantitative methods it might be well to observe the precaution of removing the skins.

Qualitative and semiquantitative methods for the estimation of peroxidase activity have been recommended on the principle that peroxidase is a very heat-resistant enzyme, and therefore permits a greater margin of safety in blanching. In principle these methods have as their basis the oxidation of certain substances in the presence of peroxidase and hydrogen peroxide. In the case of some of these substrates the oxidation results in the formation of colored compounds. Typical substrates forming colored compounds are gum guaiac, guaiacol, and benzidine. The use of these substances requires the elimination of skin tissue as with catalase; otherwise false positive reactions will be obtained as has been pointed out by Mergentime (*28*) and Campbell (*7*). Masure and Campbell (*27*) have published a method for the quantitative and semiquantitative estimation of peroxidase for application as a criterion of adequate blanching as related to frozen vegetables. The semiquantitative technique is well adapted to production control because of its rapidity and simplicity. It is based upon the use of guaiacol as the substrate and the time required for the first appearance of color in the reaction mixture. Color formation within

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch004

3.5 minutes is a positive reaction. Any color formation after this period is considered to be of no significance.

This method has been successfully applied to production-line control for over 2 years, without a single case of quality deterioration attributable to underblanching in products exhibiting a negative test during production.

However, unless the test is applied very soon after blanching, the results obtained may lead to misinterpretation and unnecessary product rejection. Peas and other vegetables yielding negative tests soon after blanching have been observed on subsequent standing and prior to freezing to yield positive reactions. This condition has been observed to carry over in the frozen and stored product.

Hand (*12*) has observed that under some conditions peroxidase upon inactivation is subject to so-called regeneration. Joslyn and Marsh (*17*), without further elaboration, stated that blanching studies were complicated by peroxidase regeneration. Subsequently Joslyn (*16*) stated that there is no indication that significant regeneration of peroxidase occurs in vegetables during freezing storage or contributes to the formation of off-flavors. In using Joslyn's method for peroxidase estimation, the writer has repeatedly observed the same increased rate of reaction with delay after blanching and in tests made after freezing. Woodroof and co-workers (*40*) reported the same reaction with snap beans, using gum guaiac as the substrate. Experience with the methods described by Lucas and Bailey (*23*) and by Davis (*8*) has indicated that they are subject to the same thing.

One might well question the idea that such observations are the result of peroxidase regeneration. During blanching certain inhibiting substances may be formed which are readily lost on standing after blanching; immediately after blanching the activity rate of the residual peroxidase present is inhibited, while with dissipation of such substances on standing the activity rate is no longer inhibited.

Experience leads one to agree with Joslyn's conclusion that this so-called regeneration does not contribute to quality deterioration. The writer has repeatedly observed with frozen pea samples, out in the trade for a year and a half and accepted at the time of packing as passing the peroxidase test and still of normal quality, positive reaction times well within the 3.5-minute time limit; very frequently the reaction time has been only a matter of seconds. If it should be proved that these observations are the result of enzyme regeneration, this is additional evidence that peroxidase is not responsible for quality deterioration in underblanched products.

Regardless of the reasons involved, it is clear that methods now available for peroxidase estimation should not be employed for establishing the adequacy of blanching in the frozen product. There is nothing in the literature to indicate that catalase behaves like peroxidase with respect to so-called regeneration, and the writer has never observed it.

With some products, particularly snap beans, there may be some reason to question the validity of a negative catalase reaction as a criterion of adequate blanching, especially in the light of the work reported by Bedford and Joslyn (*3*). In the case of peas, however, it seems to be entirely adequate. There is urgent need for investigation and the development of an adequate method for testing adequacy of blanching in finished products.

Because overblanching may result in undesirable changes in color, flavor, taste, and texture and the loss of nutritive value, it is as important to avoid overblanching as underblanching. The availability of a method for the detection of overblanching is indicated, but so far as the writer is aware, none exists at the present time. In view of the fact that complete peroxidase inactivation is not required for quality protection, a measurement of residual peroxidase activity might provide the basis for such a test.

The more dense, more mature peas sink and are drawn off near the bottom of the separator, while the less dense, less mature peas float and are carried off at the top. During the process the peas absorb salt, and unless it is removed by thorough washing enough may be retained to make the product objectionable to the taste. In a packing specification it is necessary to state the amount of salt that will be tolerated. Available methods for the determination of salt are not applicable to production-line control. One which is sufficiently simple and rapid for this purpose is definitely needed.

Finished Product

Because of their highly perishable nature, frozen fruits and vegetables are very sensitive to mishandling during distribution, especially in connection with maintaining proper conditions of temperature.

A typical example of a complaint concerning the quality of frozen peas is the presence of abnormal color, which, instead of the bright green normally expected, may be characterized as a dull olive or brownish green. This condition generally arises from underblanching or long storage at an elevated product temperature, or a combination of both. The first is definitely the responsibility of the packer; the second may be traced back to the packer, but is usually due to mishandling by the distributor.

In such an instance it is important to fix the responsibility definitely. So far as peas are concerned, the question may be effectively settled by testing the samples for catalase activity. The absence of positive catalase activity should relieve the packer of responsibility with regard to his processing technique and warrant the conclusion that the product has been subjected to elevated temperature conditions.

There are no objective tests which will enable one to tell at what point mishandling in storage took place. The packer who fails to keep a record of the quality and condition of his product as it is placed in storage, of the storage conditions, and of product condition as it is loaded for shipment is exposing himself to unnecessary risk. Without such records he may find himself unable to refute unjust or misdirected claims initiated by the buyer.

The distributor is likewise exposing himself to risk if he fails to make an inspection for quality and condition as the merchandise is received. Without this he may find it difficult to press a claim against the packer or shipping agent for damage, discovered at some later date. For protection against damage arising out of mishandling by the warehouse storing his product, he should specify the conditions under which it is to be stored, and should make periodic inspections to see that such instructions are followed. All this is very definitely a part of quality control.

It is desirable for the record to have an objective statement of the nature and degree of color deterioration. The simplest, but least desirable, method is comparison of sample color with color charts or plates such as those used in the Munsell system, Ridgeway's color standards, or the Maerz and Paul dictionary of color. Such a method is limited in value because of the difficulty of obtaining true color matches, and because of variations due to human error. The use of color charts or plates may be much improved in the Munsell system by employing a disk colorimeter (*29*). Kramer and Smith (*21*) have pointed out that the results obtained in its application to foods are sometimes difficult to explain and compare, and that the method requires special training of the operator and is tedious and cumbersome.

Spectrophotometric analysis of the color of suitably prepared extracts of the material in question often provides an excellent means of color notation. It is the least influenced by the human element, does not require special training, and is easily and quickly carried out. Kramer and Smith (*19, 21*) have found it useful for the measurement of color in certain canned vegetables and fruits. Sondheimer and Kertesz (*35*) have used it in connection with color measurement in strawberries and strawberry products. Mackinney and Weast (*24*) have employed it in analyzing color changes in frozen peas and snap beans. In many instances the method is capable not only of yielding a quantitative measure of color but also of indicating the nature of the color change. It has been shown that the color change in frozen peas as the result of high-temperature storage and underblanching is due to the degradation of chlorophyll to pheophytin [Campbell (*5*) and Mackinney and Weast (*24*)]. Spectrophotometric analysis enables one to detect the presence of this degradation product. In this regard the writer has found the method very useful. Thus the presence of pheophytin requires consideration of underblanching or high temperature storage as the cause of color deterioration. The absence of pheophytin in the presence of abnormal color should lead one to look elsewhere for the cause.

If it seems desirable to obtain additional evidence of unfavorable storage conditions, the determination of ascorbic acid content may sometimes be of value, especially if

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch004

particularly low values are obtained. It is well known [Jenkins, Tressler, and Fitzgerald (*14*) and Gortner and co-workers (*11*)] that the storage of frozen vegetables under unfavorable temperature conditions results in marked loss of ascorbic acid. In such an instance the values must be significantly lower than the normal value for similar peas processed in the same manner and properly stored.

Many objective methods of analysis may be applied to the finished product for the purpose of establishing or refuting claims concerning quality and condition, if sufficient information is available for the correct interpretation of results in terms of quality. Thus, physical methods for the measurement of texture, chemical and physical methods for maturity, spectrophotometric analyses of color, analysis of enzyme activity for condition and probable storage life, and sugar analysis for establishing taste characteristics with respect to degree of sweetness may be applied to advantage.

Included in the above list should be a method for estimating flavor deterioration and degree of desiccation and tests for estimating the adequacy of blanching in the frozen products in which one could have absolute confidence. Suitable methods are not now available; their development would be a useful and valuable field for research.

Inasmuch as ascorbic acid is affected to a marked degree in frozen products by delay in handling between harvesting and processing, under- and overblanching, inadequate cooking after blanching, excessive water fluming, inadequate storage temperatures, and length of the storage period, ascorbic acid content should be a good index of over-all quality and condition. The possibilities here have been discussed by Hohl (*13*).

In order to use any of the results obtained by objective methods as the basis for the acceptance or rejection of a product, there must be available reliable information as to the relationship between the values obtained and organoleptic quality in terms of consumer acceptance and utility. Where standards are based upon measurement of such labile constituents as ascorbic acid or sugar, a knowledge of the normal values for good commercial practice is necessary. Such values have not yet been adequately established. This should constitute a useful field for research of inestimable value to the industry.

A successful program of quality control also involves maintenance of sanitary conditions and production of products free from adulteration, contamination, and filth. Methods given by the Association of Official Agricultural Chemists (*1*) should be applied to the finished product to ensure against seizure and prosecution by federal and state food and drug authorities. In many instances such methods of analysis are not adaptable to production-line control and less accurate but more rapid methods must be substituted. With such procedures, more severe tolerances must be used to provide a sufficient margin of safety.

Conclusion

A broader outline of the scope and extent of the problems connected with quality control in the frozen food industry is given in two excellent papers by Diehl (*9*, *10*).

The development of objective methods is not difficult, and the basic procedures are available. The difficulty resides in establishing the proper relationships between analytical results and quality and condition in terms of consumer acceptance and utility, and in terms of the results to be expected in good, economical, commercial practice.

Much valuable research has been devoted to developing the basic principles for the production of frozen fruits and vegetables of high and uniform quality. If this knowledge could be applied to its fullest extent, there would be little need for concern over the quality of such foods. Before this can be done, those responsible for quality control must be provided with suitable standards of quality and condition, and objective methods of analysis which will clearly indicate conformance or nonconformance to the standards. Responsibility for this resides with the research food technologist or chemist. It constitutes a rich field for profitable and practical research.

There is yet much to be done in establishing standards and objective methods in which one may have complete confidence. The frozen fruit and vegetable industry has developed rather rapidly during the past 20 years; and it is now time for individuals and organizations responsible for the conduct of frozen food research to concentrate

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch004

seriously upon the development of adequate standards and objective methods for quality measurement. There is likewise a need for placing such standards and methods on a uniform basis the country over, so that they may have official and legal status.

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch004

Determination of Water in Some Dehydrated Foods

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Direct or indirect methods may be used to determine moisture in dehydrated foods. Indirect methods must be calibrated in terms of direct methods—the most common of which are the oven, distillation, and Fischer methods. Accuracy of the direct methods is difficult to evaluate except by comparison with a chosen reference method. Several reference methods are reviewed, but none can be given an unqualified recommendation as most practical and suitable for all foods. An indirect measure of moisture is the equilibrium vapor pressure of water, which can be measured easily and accurately. Arguments are presented to show that vapor pressure may be a better index of the stability of dehydrated foods than the moisture content, which has been frequently used for this purpose.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

Moisture determination is probably one of the most important and most widely used analytical measurements in the processing and testing of food products. It is of economic importance both to the consumer and to the food technologist. To the technologist, the moisture content is frequently an index of stability and quality of food, while to the consumer, it may serve as a measure of quantity as well as a measure of quality.

Though the literature is replete with methods of measuring the moisture content, truly accurate as well as practical methods are virtually nonexistent in the food field. The situation is well illustrated in what is probably the best compendium on this subject, the "Official and Tentative Methods of Analysis" of the Association of Official Agricultural Chemists (*2*). It becomes apparent from an examination of this volume that the stress is laid not so much on accuracy as on reproducibility and practicability of a method. Though these last two factors are for the most part the only ones of importance in the control of processing procedures and in standardization of products of commerce, the factor of accuracy is, nevertheless, of extreme importance to the research worker who endeavors to establish broad quantitative generalizations for the conditions that govern the stability of foods.

It is known that moisture plays an important role in many of the deteriorative reactions occurring in foods. These reactions can be quantitatively described only through an accurate knowledge of the moisture content. This fact was shown in studies on browning of dehydrated vegetables (*15*, *27*) and fruits (*33*) and on oxygen absorption by egg powders (*22*). With the vegetables, for example, the rate of browning was found to vary exponentially with the moisture content. Accuracy is also important in many industrial problems. Moisture content must be known accurately in the evaluation of "materials balance" or of processing losses, and in the comparison of moisture determinations by different laboratories and by different methods. The requirement of accuracy is not nearly so severe for fresh foods of high moisture content (fruits and vege-

In ANALYTICAL METHODS IN THE FOOD INDUSTRY;
Advances in Chemistry; American Chemical Society: Washington, DC, 1950.

tables) as for dehydrated ones (10% moisture or less), where a small absolute error in measurement represents an appreciable fraction of the total water.

In a search for an accurate method of measuring moisture in foods, one cannot overlook the essential requirements of convenience, speed, and precision. Many currently used methods meet these requirements without necessarily yielding accurate results under the conditions used. Probably most important are the electrical methods (*13, 24, 25, 35*), the air- and vacuum-oven methods (*1, 2, 6, 18, 25, 28, 35*), distillation with organic solvents (*1, 3, 7, 12, 13, 25, 35*), and the Karl Fischer reagent method (*9, 11, 25, 31, 32*). Without discussing the relative merits of these methods, it can be assumed that accurate results could be obtained with each method by calibration against some accurate reference method.

It appears then that the establishment of practicable and accurate reference methods presents one of the major problems in the determination of moisture in foods. The present paper summarizes some of the important factors that govern the accuracy of moisture determinations in general, and reviews some of the recently published attempts to develop methods that might serve as useful reference standards for dehydrated foods, and especially for dehydrated vegetables and fruits.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

General Considerations in Determination of Moisture in Dehydrated Foods

The analytical procedures used in the determination of moisture can be divided for convenience into two general classes, the direct and the indirect. In the direct methods an attempt is made to remove all the water from the sample and to determine its quantity by measuring the loss of weight of the sample, by collecting the water and measuring its volume, or by extracting the water and causing it to react quantitatively with some reagent. The indirect methods do not require removal of water, but depend on the variation with moisture content of some physical or physicochemical property of the materials, such as electrical conductivity (*13, 24*), dielectric constant (*13*), or vapor pressure (*8, 20, 37*). The methods in this class must, however, be calibrated by means of one of the direct methods. The calibration may vary with method of processing or source of the food because of possible differences in composition.

In choosing a reference method for the calibration of others, one is therefore restricted to direct methods. For this reason consideration is given here to some of the assumptions that underlie the important methods of this class.

Because of their wide applicability, emphasis is laid on the distillation methods, the Karl Fischer reagent method and particularly the vacuum-oven method. The principal assumptions are as follows:

Water is the only volatile component of the material.

The effect of chemical side reactions that may occur during the removal of water is negligible.

The removal of water from the material is essentially complete.

Water the Only Volatile Component. This assumption pertains especially to the various oven methods. The magnitude of the errors caused by the volatility of nonaqueous components will depend on the composition of the food, the temperature of drying, and the pressure maintained in the oven.

In studies on the moisture determination of corn by vacuum drying at 100° C., Sair and Fetzer (*28*) found that an error of about 0.5% in moisture content could be attributed to volatility of oils. The error was negligible in vacuum drying at 70° C.

Similar information for other foods would be of great value. With dehydrated vegetables and fruits, large errors are not to be expected, because these foods do not normally contain much nonaqueous volatile matter and some of it will have been lost in the process of dehydration.

Possible Effects of Chemical Side Reactions during Removal of Water. The types of reactions that are likely to occur are thermal decomposition of unstable components, or an interaction among the components. The reactions may result in the

formation of additional water or of other volatile substances. Any analytical method which requires that the sample be brought to an elevated temperature is subject to error from these sources. Such errors may occur in all the oven methods, the distillation method, and the Fischer method. Although these errors may be reduced to a negligible value by removing water at sufficiently low temperatures, there is no way of foretelling the "safe temperature" for any particular substance. Probably the only guide that has been used for fruits and vegetables is based on the observation that fructose (a common and not very stable constituent) undergoes but little decomposition when it is heated at temperatures not higher than 70° C. (*10*).

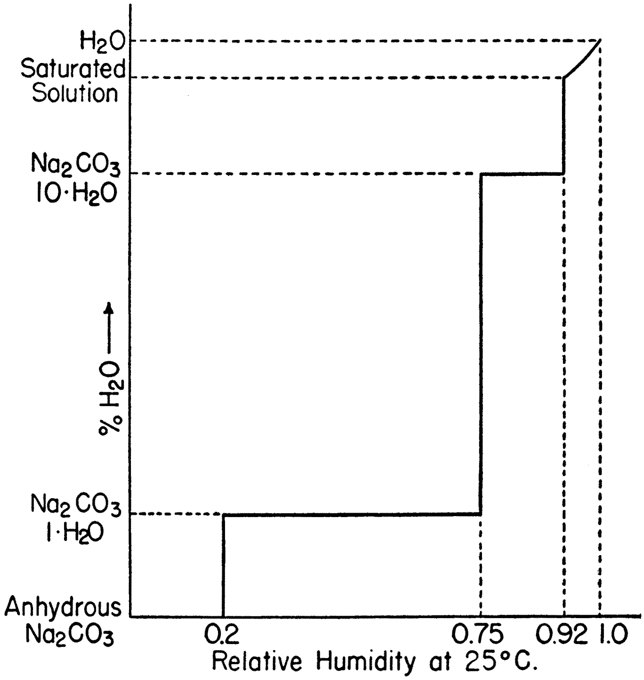


Figure 1. Isotherm for Sodium Carbonate (30)

Another interfering chemical reaction is that of oxygen of the air with oxidizable components of the food material. This error would be greatest in air-oven drying and is probably not appreciable when the drying is conducted in an inert atmosphere or in vacuo. In the Fischer method there are other sources of error. The Fischer reagent may react with substances other than water, such as ascorbic acid or other iodine-reducing substances (*11*), or additional water may be formed through combination of the commonly used solvent methanol with carboxyl or carbonyl substances in the sample (*25, 32*).

Essentially Complete Removal of Water from Sample. Though this assumption must be made in the case of all direct methods (oven, distillation, Fischer, etc.) its correctness is most difficult to prove and in many cases it turns out to be one of the greatest sources of error. Two factors are involved: the equilibrium moisture content and the rate of removal of moisture.

EQUILIBRIUM MOISTURE CONTENT. The difficulties in removing water from most foods can best be understood by considering the nature of the component substances and the manner in which water is held in them. The most important components such as starches, pectins, cellulose, proteins, and sugars are for the most part colloidal substances of high molecular weight. It is now generally recognized that water is held in these substances by forces of adsorption which are usually attributed to van der Waals forces or to hydrogen bond formation (*38*). In contrast with the crystalline hydrates,

which release their water of crystallization when the water pressure in the surrounding atmosphere is reduced to some finite value, the equilibrium amount of water adsorbed by the colloidal substances merely decreases gradually with decreasing pressure, and the forces that hold the remaining water actually become stronger with decreasing moisture content (*4*).

The relation between moisture content and vapor pressure (or relative humidity) at constant temperature is expressed by an isotherm. Figures 1 and 2 show typical isotherms for a crystalline hydrate sodium carbonate and a food material (potato).

It is seen from Figure 1 that sodium carbonate will become anhydrous (neglecting physically adsorbed water) when the relative humidity of surrounding atmosphere is reduced to 0.2. In the case of the potato, it has been estimated (*17*) that even when the relative humidity is reduced to 0.005, the remaining moisture is still appreciable—about 1%.

The fact that the equilibrium moisture content may be considerable at low humidities is of especial importance in the oven methods. Under ideal conditions no water vapor should be present in the oven, but this is impossible to attain in practice. It is difficult to maintain a dry atmosphere in an air oven, and most commercial vacuum ovens are not air-tight. Thus, the discrepancies in results of different investigators have frequently been traced to different humidities in their ovens. Any attempt to reduce the relative humidity by increasing the oven temperature introduces the danger of error from thermal decomposition.

It is difficult to estimate the magnitude of the error due to insufficiently low humidity when distillation methods are used with organic liquids such as toluene (*6, 28*), xylene (*6, 28*), or chloroform (*12*). With organic liquids essentially immiscible with water and of high boiling point the error is probably very small. When methanol is used as an extraction solvent, as in the Fischer reagent method, the amount of unextracted water is undoubtedly some function of the concentration of water in the alcohol, but the error might be small because of substitution of adsorbed water by adsorbed alcohol (*23, 34*). This seems to be borne out by experiments of Schroeder and Nair (*31*), who deliberately added water to the alcohol to form a 0.5% water solution and found that the results of their moisture determinations were essentially the same as with "anhydrous" methanol, which contained about 0.05% water.

RATE OF REMOVAL OF MOISTURE. The factor that contributes most significantly to the difficulties of complete removal of water is the extreme slowness of diffusion of water vapor through the shrunken, dense, and glasslike solid gel that characterizes most dehydrated foods. When the moisture content falls below approximately 10%, the rate of diffusion decreases not only because of rapidly decreasing vapor pressure (see Figure 2), but, more significantly, because of the very rapid decrease in diffusion coefficient with decreasing moisture content. It has been shown (*36*) that the rate of drying becomes virtually independent of the rate at which water is removed from the surface of the material (as by increasing air flow in air drying, decreasing pressure in vacuum drying, or increasing distillation rate in distillation methods with organic liquids), but is determined almost entirely by diffusion from within the pieces of the material.

Table I. Variation of Apparent Moisture Content of Dehydrated Carrots with Particle Size and Time of Drying in Vacuum Oven at 70° C.[a]

	Loss of Weight			
	As Apparent % H_2O at Various Drying Times			As % H_2O after regrinding to 40-mesh[c], 44 hours
Particle-Size Distribution[b]	6 hours	22 hours	44 hours	
Passes 5-mesh and retained on 10-mesh	2.7	4.3	5.2	6.5
Passes 10-mesh and retained on 18-mesh	3.2	4.6	5.6	6.3
Passes 18-mesh and retained on 35-mesh	3.9	5.2	5.9	6.2
Passes 35-mesh	5.2	5.9	6.1	6.2
Maximum differences among different fractions	2.5	1.6	0.9	0.3

[a] Data by Makower and Myers (*20*).
[b] Distributions prepared from single lot of diced dehydrated carrots by grinding through food chopper and separating ground material into fractions by sieves.
[c] Each fraction ground again to pass 40-mesh sieve before drying.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

Many hours are therefore required to effect virtually complete removal of moisture. The actual time depends on the nature of the material, the temperature, and the particle size. The time may, of course, be shortened by fine grinding to reduce the particle size and by operating at a high temperature. There are, however, practical limitations to this procedure.

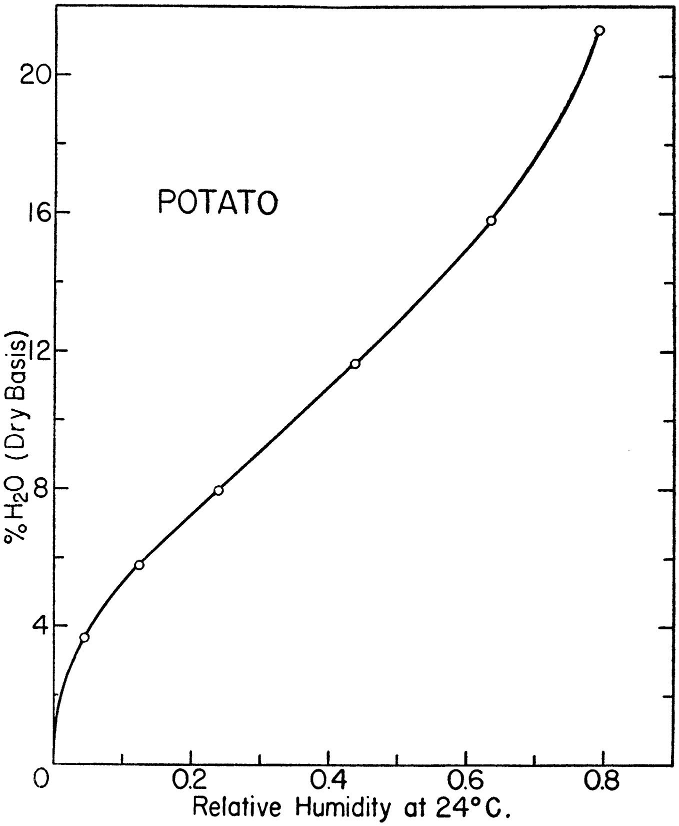


Figure 2. Isotherm for Potatoes (*17*)

Grinding beyond a certain limit requires an objectionably long time, with consequent overheating, possible loss of moisture, and in some cases the formation of a gummy caked mass. This subject has been treated in some detail by Makower, Chastain, and Nielsen (*18*), who decided that an optimum comminution is obtained in grinding through a 40-mesh sieve. They employed a Wiley-type mill which has the advantage of protecting the ground material from undue exposure to the air. Grinding gives rise to a distribution of particle sizes, the largest particles of which are the slowest to dry. Size distribution of the particles and moisture distribution among the particles may also vary with the over-all moisture content, with the distribution of moisture in the unground material, and with the type of material. Reproducibility of moisture determinations by the direct methods depends, in part at least, on the reproducibility of grinding.

As an illustration, the effects of varying the particle size distribution, and of temperature, on the course of water removal from dehydrated carrots in a vacuum oven are shown, respectively, in Table I and Figure 3.

Data in Table I reveal that sieved fractions of different particle size distribution lose varying amounts of moisture during the same drying period at 70° C. The last column in the table shows that the apparent percentage of moisture in the different frac-

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tions was approximately the same (maximum difference 0.3%). These results were obtained by regrinding each fraction through a 40-mesh sieve, followed by drying for 44 hours. The discrepancy in the weight losses (or apparent moisture content), shown in the last row of Table I, is greatest for the 6-hour period which was the commonly employed drying time for dehydrated fruits and vegetables (*1*). Even at the longest drying time, 44 hours, there was still an appreciable difference of 0.9%.

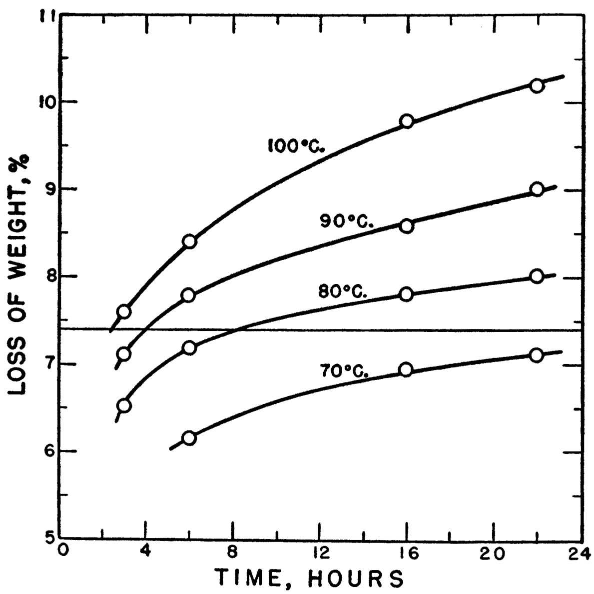


Figure 3. Loss in Weight *vs.* Drying Time for Carrots at Various Temperatures

Moisture content determined by redrying procedure at 70° C., indicated by horizontal line crossing ordinate at 7.4%. 70° C. curve intersects horizontal line at approximately 35 hours (not shown in graph; see Table IV in *18*)

Increase in temperature to hasten removal of water leads to complications due to increase in rate of decomposition of unstable substances. This is illustrated in Figure 3, which shows the results of drying dehydrated carrots at various temperatures. The moisture content of the carrots, estimated by means of the redrying procedure (*18*) explained below, is shown by a horizontal line, and the intersection of this line with the various drying curves indicates the required drying times at various temperatures. The time varies from about 35 hours at 70° C. to about 3 hours at 100° C. It is also evident from this figure that considerable decomposition occurs at the elevated temperatures and that great care must be exercised in accurately controlling the temperature in order to get reproducible results. The means of establishing an accurate, uniform, and reproducible temperature in a vacuum oven have been described (*18*).

The considerations described above for vacuum-oven drying hold equally well when water is removed by distillation with an organic liquid, as exemplified by the Bidwell-Sterling method (*3*). The time of distillation for various dehydrated vegetables has been shown to depend on particle size and temperature (*13*) and is about the same as would be required in vacuum-oven drying under the same conditions (*28*).

When the foregoing considerations are applied to the Fischer method it is rather surprising to note that the time for essentially complete removal of water is, in general, very much shorter than that required in a vacuum oven and that in many cases the particle size does not seem to be of great importance (*11*). Thus, for example, a 0.5-hour extrac-

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tion of carrots (40-mesh) in methanol at 60° C. yields results approximately equivalent to 40 or 50 hours of drying in a vacuum oven at 70° C.

The explanation must lie in the difference of permeability to water when the food material is soaked and extracted in alcohol. It is known that removal of water from colloidal substances of high molecular weight results in a shrinkage and collapse of the structural lattice (*26*, *34*). The shrinkage probably hinders the escape of the remaining water. In the presence of alcohol, which replaces the adsorbed water (*23*, *34*), this collapse is probably prevented or is less pronounced, so that the water diffuses out more readily. Furthermore, the alcohol dissolves or leaches out certain components of the food tissue—e.g., sugars—leaving the tissue porous and more permeable. Some supporting evidence for the effect of solubility may be had from the rather unique behavior of white potatoes as observed by Johnson (*11*). Having a low sugar content, potatoes required a 6-hour extraction time as compared to less than 0.5 hour for most foods high in sugar or in other soluble substances. This behavior is illustrated in Figure 4, which shows the effect of extraction time on the apparent moisture content of various dehydrated foods.

It has also been found by Sair and Fetzer (*28*) that extraction of certain solubles from corn by treatment with alcohol materially hastens the speed of drying in a vacuum oven.

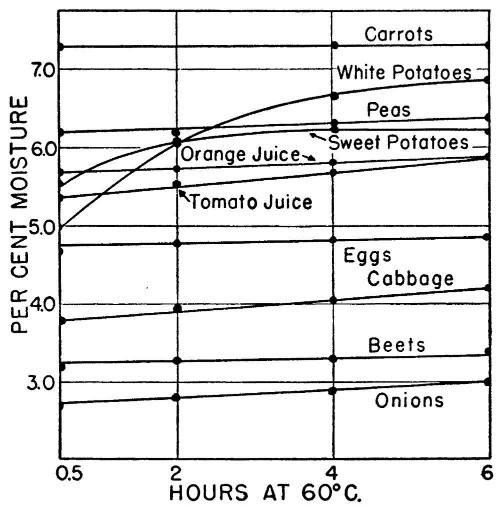


Figure 4. Moisture Determinations with Fischer Reagent as Function of Time of Soaking in Methanol at 60° C. (*11*)

Makower and Nielsen (*21*) have described another method that can be used to alter the texture of the dehydrated material in order to increase its permeability to water vapor and to shorten the time required for removal of water. The method, called the "lyophilization procedure," involves the following steps:

> A large amount of water is added to the dehydrated material in order to cause it to swell; the swollen structure is preserved when the material is frozen and subsequently dried in vacuo (in the frozen state) to a low moisture content. Some leaching occurs during the treatment with water and this, undoubtedly, further contributes to the increase in the porosity of the solid. Drying of the lyophilized substance can be completed in a relatively short time in a vacuum oven at an elevated temperature, or at room temperature in the presence of an efficient water adsorbent.

The efficacy of lyophilization is demonstrated by the fact that the speed of drying of diced carrots (approximately 0.5-cm. cubes) in a vacuum oven was much greater than that

of the same carrots, unlyophilized but ground to pass a 40-mesh sieve. The lyophilization procedure is, therefore, equivalent to grinding to a fineness which usually cannot be practicably attained with the unlyophilized substances.

Recent Attempts to Develop Accurate Moisture Methods

Redrying Procedure. Makower, Chastain, and Nielsen (*18*) described a method called the "redrying procedure" for the determination of the drying conditions to be used in the measurement of the moisture content of dehydrated vegetables by the vacuum-oven method. At the time this work was started, no standard method existed, and all dehydrated vegetables were tested alike—namely, by an A.O.A.C. method (*1*) originated for dehydrated fruits. The moisture content was taken to be the loss of weight of material ground in a food chopper and vacuum-dried at 70° C. for 6 hours. With carrots, for example, it is obvious (Figure 3 and Table I) that the 6-hour time is purely arbitrary and that the correct time cannot be picked from a drying curve. It is impossible to tell when the water is completely driven off and when further loss in weight may be ascribed to degradation of material. There is no sharp demarcation, and chemical changes probably occur at all stages.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

Table II. Comparison of Redrying Procedure with Primary Reference Method[a]

Material	Redrying Procedure: Drying time, hours	Drying temperature, ° C.	Loss in weight, % H_2O	Reference Method[b]: Loss in weight, % H_2O
Carrots				
Chantenay I	30	70	7.8	7.7
Chantenay II	35	70	7.2	7.0
Imperator	30	70	4.3	4.1
Potatoes				
White Rose				
Low sugar	50	70	8.4	8.6
High sugar	35	70	9.5	9.5
Klamath Russet	45	70	7.8	8.0
Cabbage				
Flat Dutch	30	60	3.9	3.8
Onions				
Yellow Spanish	30	60	6.0	6.1

[a] Data by Makower *et al.* (*18*). All measurements made on materials ground to pass 40-mesh sieve.
[b] Drying to constant weight at room temperature, in vacuo, over $Mg(ClO_4)_2$. Carrots and potatoes dried 11 months, cabbage and onions 9 months.

The problem was, then, to determine the correct time of drying at a specified temperature by calibration against some reference method. As indicated above, it was also necessary to establish reproducible methods of sampling and grinding of the vegetable, and of maintaining in the oven a uniform temperature and a low pressure of water vapor, and to consider other experimental factors (*18*).

The chosen reference method was defined as follows:

The ground material (40-mesh) is dried in vacuo at room temperature over a desiccant (magnesium perchlorate) that permits practically no water vapor pressure. An assumption was made that at room temperature and in the absence of air, decomposition and oxidation would be negligible. It was found, however, that a direct application of this reference method was not practicable from a routine standpoint, because the time to reach equilibrium was exceedingly long (6 to 9 months).

A secondary reference method known as the redrying procedure was therefore established for a more rapid calibration.

A sample ground to 40-mesh is dried in the oven for a long time (100 hours or more) at a temperature where decomposition is not too rapid; at the end of this time the sample is assumed to be essentially dry. A drying curve is determined from the loss of weight at various time intervals. The sample is then permitted to absorb in a humidistat a known amount of water which can be accurately determined by weighing before and after the absorption. Drying is then repeated under conditions identical with those of the

first time, and a second drying curve is determined. The time required in the second drying to obtain a weight loss (moisture loss plus decomposition loss) equal to the amount of added water is taken as the correct drying time for this material.

An illustration of the results obtained for carrots is shown in Figure 5. The amount of water added to the sample prior to the second drying was 5.3%. The time required to remove this amount of water, as indicated by the vertical line at point T_2 on the second drying curve, was 30 hours. At the corresponding time, T_1, on the first drying curve, the weight loss was 4.3%. This percentage is then taken to be the moisture content of the original sample before the first drying.

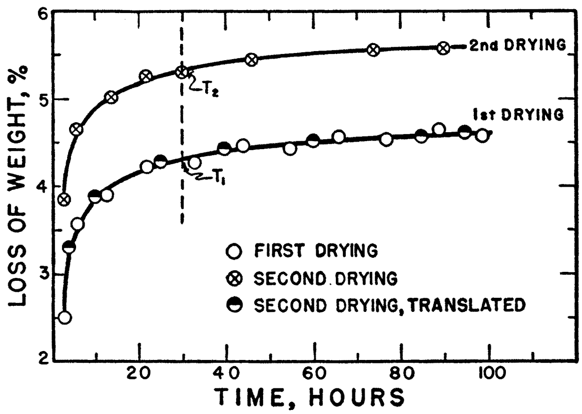


Figure 5. Drying and Redrying Curves for Carrots at 70° C. (*18*)

The assumption that the sample was essentially dry at the end of the first drying run was verified by the fact that drying the first time for an additional 50 hours resulted in the same redrying time. It is also assumed that the course of drying (rate and amount of decomposition) was the same in both dryings. This is verified by the fact that the two curves are identical in shape (superposable), as shown in Figure 5 by translation of the experimental points through equal distance from the second curve to the first.

If too much decomposition occurs at the chosen temperature, the course of the two dryings will not be the same and it is then necessary to operate at a lower temperature. This necessity is shown in Figure 6 for cabbage, where it is seen that 70° C. curves are not superposable, while those for 60° C. are.

Comparison of the results obtained by the redrying procedure with those by the primary reference method (room temperature, in vacuo over magnesium perchlorate) is shown in Table II. The agreement of the two sets of results was within 0.2% or better, which is considered satisfactory and serves to confirm the suitability of the redrying procedure as a secondary reference standard.

It is evident from Table II that somewhat different drying times are required for different varieties or compositions of a vegetable. These differences are, however, not of great importance in routine measurements of moisture where an error of ±0.1% is usually permissible. By making allowances for this error, permissible ranges of drying time could be established for several vegetables from calibrations on different varieties at various moisture contents (Figure 3 in *18*). These ranges for drying at 70° C. were as follows: carrots, 29 to 35 hours; potatoes, 43 to 67 hours; cabbage, 7 to 9 hours; onions 5 to 8 hours. Drying times at other temperatures could be determined empirically as shown in Figure 3.

It is realized that such rigorous assignment of drying times cannot be strictly correct, because the required time should increase with increasing moisture content. The errors thus incurred would, however, be small for most dehydrated vegetables (moisture

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range of 3 to 10% water), because most of the water is driven off in the first hour or two, and the major part of the long drying period is necessary to remove the remaining 1 or 2% of the water. If the initial moisture content of the sample were below 2%, the assigned drying times would be unnecessarily long but the error involved would still be negligibly small (Figure 3 of *18*).

Lyophilization Procedure. Makower and Nielsen (*21*) demonstrated that a great simplification and a saving of time in the calibration can be achieved by the application of the lyophilization procedure, described above. Because of the remarkable increase in the drying rate produced by this method, it was possible in some cases to make direct use of the primary reference method (room temperature drying in vacuo over magnesium perchlorate) as a calibration method. For example, with lyophilized sweet potatoes and white potatoes, a constant weight in drying was reached in 11 and 4 days, respectively, whereas the same materials in unlyophilized state required a drying time of over 6 months.

With materials rich in sugar (beets and carrots) room temperature drying was considerably longer, amounting to several weeks. In these instances (as well as with white and sweet potatoes), an unambiguously constant loss of weight was obtained in a short drying at some elevated temperature, 60° or 70° C. Moreover, the loss of weight so obtained was the same (within 0.1%) as that at room temperature with the primary reference method. It was concluded, therefore, that the rapid drying of lyophilized materials at elevated temperatures could be used as a satisfactory secondary reference method.

A comparison of the drying curves for lyophilized and unlyophilized sweet potatoes is shown in Figure 7. It is seen that the same and constant loss of weight (8.2%) was reached with the lyophilized samples at both 60° and 70° C. No such indication is shown by the unlyophilized samples at either temperature.

Of the four lyophilized vegetables that were investigated, only carrots failed to reach a constant weight in drying for 140 hours at 70° C. Constancy was, however, obtained in 100 hours at 60° C. It was concluded that the decomposition rate for this vegetable was appreciable at 70° but not at 60° C. The drying times required to attain constant weight varied for the several vegetables from 20 to 40 hours at 70° C., and from 40 to 100 hours at 60° C.

It is of interest now to compare the moisture values obtained by the lyophilization procedure with those by the redrying procedure. Comparison in Table III for four vegetables shows that the results of the two independent methods agree within 0.1 or 0.2%. In view of this good agreement it seems fairly reasonable to assume that the measurements represent a very close approximation to the actual water present in the samples.

Table III. Comparison of Moisture Determinations on Lyophilized and Unlyophilized Vegetables

(% moisture, for various drying conditions)

	Lyophilized[a]			
	Vacuum desiccator over $Mg(ClO_4)_2$, room temperature	Vacuum Oven 60° C.	Vacuum Oven 70° C.	Unlyophilized[b], Vacuum Oven, 70° C.
White potatoes	8.7	8.8	8.8	8.8
Sweet potatoes	8.2	8.2	8.2	8.3
Carrots	7.2	7.5	...	7.3
Beets	6.7	6.9	6.8	6.8

[a] Moisture determined by drying to constant weight under stated conditions (*21*).
[b] Drying times obtained from calibration by redrying procedure (*18*). Times were: white potatoes, 60 hours; sweet potatoes, 120 hours; carrots, 33 hours; beets, 100 hours (*17*, *18*).

The lyophilization procedure, as described, is suitable for materials such as vegetables, that do not usually contain much nonaqueous volatile matter. For other materials, rich in these volatiles, a modification of the procedure would be required. The volatiles could in some cases be extracted with a solvent and dried separately (*28*), or the substances evolved in the drying could be collected and analyzed for the amount of nonaqueous material.

From the fact that a constant weight in drying at 70° C. is reached with some lyo-

philized materials one might assume that the decomposition rate is negligibly slow and that the continuous loss of weight observed in the corresponding unlyophilized vegetables at 70° C. is entirely due to slow diffusion of water. If that were true, the unlyophilized vegetables would eventually be expected to reach the same constant weight as those that were lyophilized. It was found, however, that the loss for the unlyophilized substances was measurably greater (Table I in *21*).

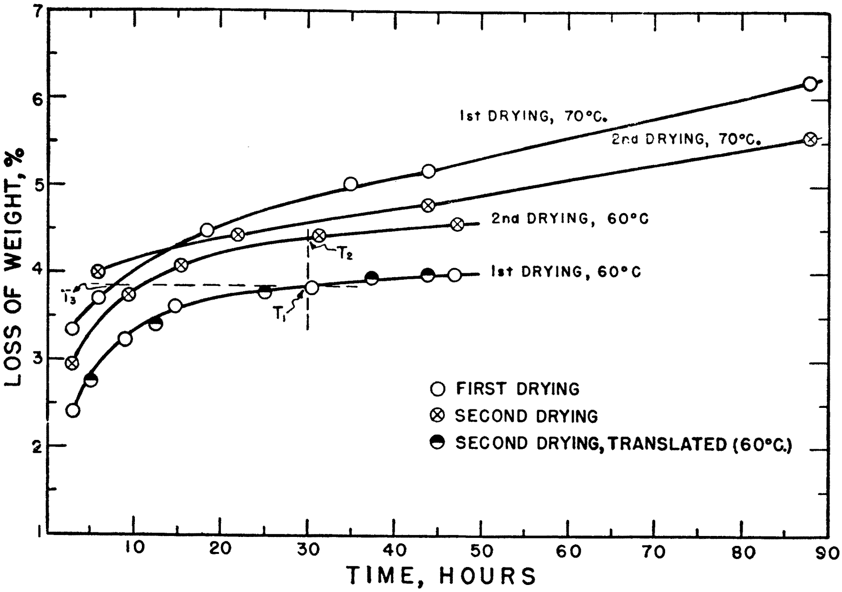


Figure 6. Drying and Redrying Curves for Cabbage at 60° and 70° C. (*18*)

60° C. curves are superposable; 70° C. curves are not. Amount of water added prior to second drying at 60° C., 4.4%; redrying time, T_2, 30 hours; moisture content of original sample, T_1, 3.9%. Drying time at 70° C., T_3, 8 hours

This observation may be interpreted on the assumption that the decomposition rate is negligible in the absence of moisture and that the rate becomes faster with increasing moisture. The unlyophilized samples would thus be expected to suffer considerable decomposition, because they dry very slowly and are therefore heated in a relatively moist state for a long time. Further support for this explanation is afforded by the observation that the apparent moisture content of certain dehydrated vegetables increases markedly in storage at elevated temperatures, and that this increase is greater at higher moistures (*17*). A similar observation was made by Sair and Fetzer (*29*) on gluten feed.

Studies on Corn. Sair and Fetzer (*28*) conducted very detailed and exhaustive investigations on the determination of moisture in dried corn. In this study they demonstrated full awareness of the various factors affecting the measurement of moisture, as described above. The measurements were made on a single and homogeneous lot of corn ground to 40-mesh, by methods that included drying in air and in vacuum ovens at various temperatures, distillation with various liquids, and the DeBruyn method, which consists of vacuum drying to constant weight over phosphorus pentoxide at 40° C.

The results obtained by the distillation methods with solvents ranging in boiling points from 80° C. for benzene to 140° C. for xylene are shown in Figure 8. The results with the various oven methods are shown in Figure 9.

Virtually identical results (within 0.1%) were obtained by distillations with toluene and benzene, and by the DeBruyn method. It is surprising to note from Figure 8 that constant values for moisture were obtained at the high distillation temperatures of 80° and

110° C. Evidently there was no decomposition and one is led to assume that corn is an unusually stable vegetable. Makower *et al.* (*18*) found considerable decomposition with most vegetables at temperatures above 70° C. The results of vacuum-oven measurements at a high temperature (110° C.) also fell into agreement after they were corrected for decomposition. The correction was estimated by means of a so-called "reversibility procedure" described below. It is not clear, however, why decomposition should have occurred in the vacuum-oven drying at 110° C. and not in the distillation with toluene at the same temperature. One might suspect that volatiles other than water were measured in the oven method. The results of distillation with xylene (140° C.) were too high because of decomposition, which was evidenced by browning of the sample. Drying in an air oven at 100° C. gave low results. This was ascribed to insufficiently low humidity in the oven atmosphere.

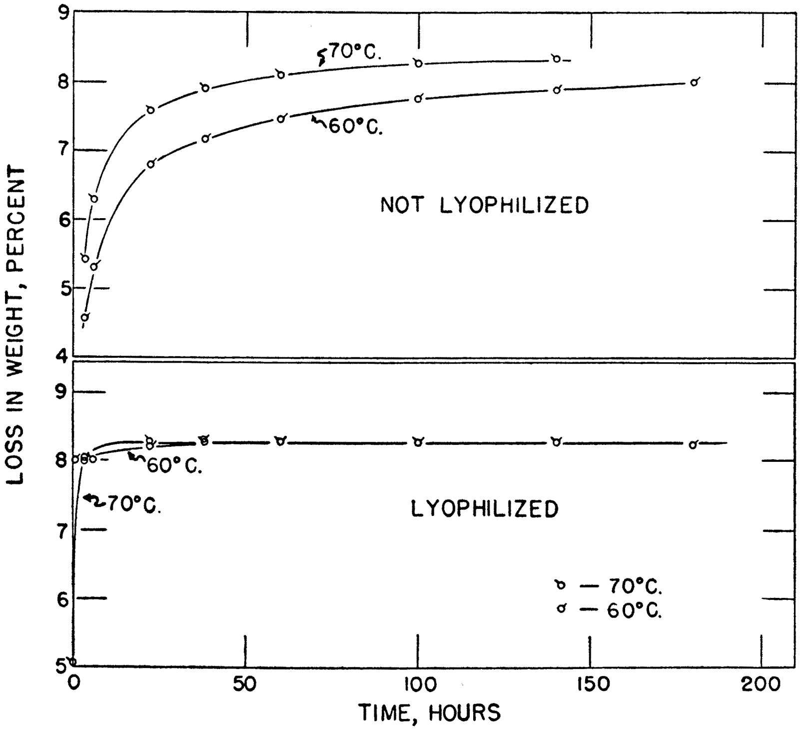


Figure 7. Drying Curves for Lyophilized and Unlyophilized Sweet Potatoes at 60° and 70° C. (*21*)

It is interesting to note from Figures 8 and 9 that the times required to remove the water from the fine powder by the various methods were very long. The time ranged from 20 hours at 110° C. for the toluene method to approximately 20 days for the DeBruyn drying at 40° C. The vacuum-oven drying at 70° C. required about 60 hours which fits excellently into the range of drying times determined by Makower *et al.* by the redrying (*18*) or lyophilization procedures (*21*) for various root vegetables ground to the same fineness (40-mesh).

The "reversibility procedure" for the estimation of the amount of decomposition that occurs in the oven drying at 110° C. was conducted as follows:

A sample dried at 110° C. was remoistened and then redried at a low temperature and a controlled low humidity to determine its equilibrium moisture content (regain). This value was compared with the regain of a remoistened control sample which was dried originally at a low temperature where decomposition was negligible (as proved by the

In ANALYTICAL METHODS IN THE FOOD INDUSTRY;
Advances in Chemistry; American Chemical Society: Washington, DC, 1950.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

same regain obtained on repeated dryings). The difference of the two regains was taken to represent the decomposition at 110° C. As pointed out above, this could represent the loss of volatiles other than water rather than decomposition. It was assumed, with certain justifications, that adsorptive capacity of the 110° C. sample had not been altered. Thus, for a sample heated for 32 hours at 110° C. decomposition was calculated to be 0.60%. On subtraction of this from the observed weight loss, a "true" moisture value was obtained which was in very good agreement with the results of other methods.

From the excellent agreement in measurements by the various methods Sair and Fetzer concluded that they have determined the true moisture content with an accuracy of approximately 0.1%. Though this claim is subject to further verification, it does indeed seem to be plausible.

Karl Fischer Reagent Method. The Fischer reagent method has been applied to many foods (*9, 11, 31*) and has gained increasing importance in recent years because of its rapidity and reproducibility. As explained above, the extraction of water from a sample of dehydrated fruit or vegetable is very rapid in boiling methanol. This makes it frequently possible to complete a determination in less than a half hour, and the results are less dependent on the fineness of grinding than with most other common methods (*11*). These advantages are often sufficient to outweigh the disadvantage of the necessity for frequent calibration of the rather unstable reagent.

It is extremely difficult to evaluate the accuracy of the method for different foods. Unfortunately, there are virtually no data available to compare the Fischer method with any of the reference methods described previously. One exception will be referred to later. Comparisons have been made by many investigators against other methods, but no conclusions could be drawn, because the accuracy of the reference methods was also unknown (*9, 11, 31*).

One example of such a comparison for several dehydrated foods against a vacuum-oven method is shown in Table IV. The data for the Fischer method are given for three different extraction times and are also shown graphically in Figure 4. The drying conditions were arbitrarily chosen to be 38 hours at 70° C.

Table IV. Comparison of Vacuum-Oven and Fischer Reagent Results for Dehydrated Foods[a]

	Apparent Moisture, %			
		Fischer Results after Different Extraction Times in MeOH at 60° C.		
Material[b]	Vacuum oven, 38 hours, 70° C.	0.5 hour	6 hours	24 hours
Carrots	6.62	7.32	7.26	7.26
Beets	3.07	3.18	3.29	3.49
Peas	6.00	6.32	6.29	6.58
Orange powder	5.71	5.71	5.76	5.81
Sweet potato	5.40	5.60	6.21	6.40
White potato	6.84	4.86	6.90	7.32
Onion	2.82	2.70	2.96	3.28
Cabbage	3.63	3.76	4.16	4.64
Tomato powder	5.40	5.30	5.76	5.81

[a] Data from Johnson (*11*).
[b] Materials ground to pass 40-mesh sieve.

The comparison is complicated by the fact that the Fischer method does not always yield an unequivocal end point. As shown in Figure 1, in many instances (potatoes, cabbage, onions) there is a gradual increase in the apparent moisture with extraction (refluxing) time. The increase is undoubtedly due in some cases to slow diffusion of water (potatoes), and in others to certain side reactions which were mentioned above. Schroeder and Nair (*31*) assumed that the increase is generally due to decomposition of the sample at the temperature of boiling methanol. Even in the cases where the result is virtually independent of the refluxing time (carrots, peas, beets) one cannot be certain that some rapid side reaction has not occurred during the course of titration. Schroeder and Nair demonstrated that the Fischer reagent is by no means specific for water. They allowed onions and protein hydrolyzate to soak in excess of the reagent and noted that there was a progressive increase in the consumption of the reagent with no indication of an end point.

The data in Table IV show that, with the exception of potatoes and carrots, the vacuum-oven results do not differ by more than 0.2 or 0.3% from the results with the Fischer method when a half-hour extraction was used. In order to obtain an approximate agreement with the oven method it is necessary to extract the potatoes for about 6 hours and the carrots for less than half an hour.

It is evident that the procedure to be used with the Fischer reagent can be established only in terms of some standard reference method. Schroeder and Nair (*31*) adopted a calibration method which involved titration with the Fischer reagent after a prolonged extraction of water from the sample in methanol at room temperature. It was assumed that the extraction at low temperature, and the avoidance of an excess of the reagent, would minimize the extent of side reactions. Two procedures were used.

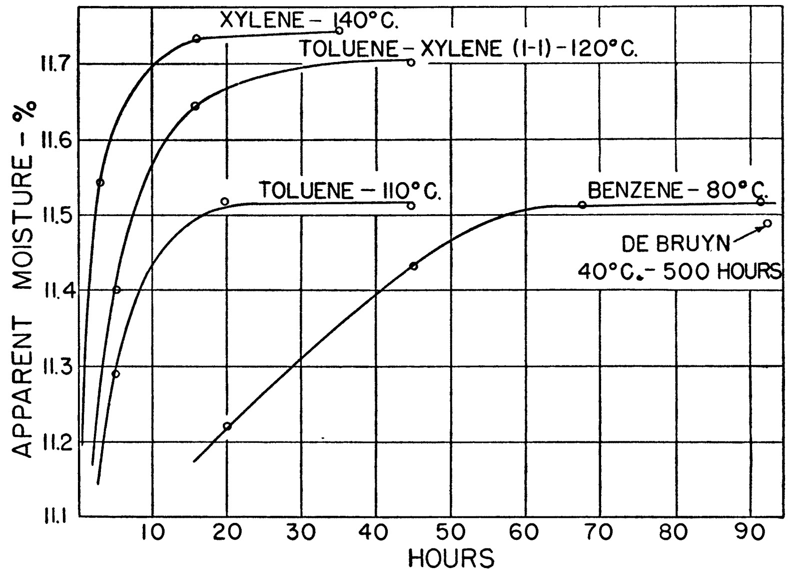


Figure 8. Determination of Moisture in Corn by Distillation Methods (28)

The intermittent method involved a repeated succession of extractions and titrations of one sample; the other involved titrations of different samples of the same material, extracted for different lengths of time, until a constant titration value was reached.

The results for the intermittent titration are shown in Figure 10. The progressive and linear increase in the apparent moisture with time, observed for most materials, was ascribed to some side reaction. The "true" moisture was obtained by extrapolation of the straight-line portion to zero time.

The extrapolated values together with results of the second calibration method are shown in Table V. With the exception of cabbage, agreement to better than 0.2% was found in the results of the two methods for the various foods. The proper refluxing time, for a rapid determination, was then selected from the data for the high temperature extraction shown in the last four columns in Table V. The required times varied from 5 minutes for onions and carrots to 30 minutes for celery. The shortness of these times as compared with other methods illustrates the potential usefulness of the Fischer method as a rapid control method, even though its accuracy is uncertain.

It can probably be assumed that a satisfactory test of the accuracy of a given method would consist of a comparison of its results with those of another independent method which is different in principle. Such a comparison is available in the case of onions (*16*). It was found that the results by the Fischer method (Schroeder and Nair calibration) were approximately 0.2% higher than those obtained by the lyophilization procedure (*21*).

Table V. Calibration Data for Fischer Reagent Method[a]

Dehydrated Materials	Low Temperature Reference Methods A[b]	B[c]	Results after Refluxing in Boiling MeOH for Various Times 2 min.	5 min.	15 min.	30 min.
			Apparent % Water			
Onion	3.95	3.97	3.90	3.90	3.84	4.01
Protein hydrolyzate	2.41	2.56	2.47	2.44	2.51	2.54
Carrots	6.13	6.22	6.13	6.20	6.26	6.32
Peppers	3.58	3.61	3.35	3.49	3.61	3.78
Cabbage	3.82	4.37	3.96	4.00	4.07	4.19
Celery	2.99	2.97	2.79	2.84	2.91	2.99
Tomato	3.20	3.25	3.04	3.07	3.16	3.32

[a] Data by Schroeder and Nair (*31*).
[b] Intermittent titration of one sample.
[c] Titration of different samples.

This good agreement may be interpreted to mean, on the basis of the above assumption, that both methods are accurate, at least for onions, to within 0.2%.

Pressure of Water Vapor as an Index of Moisture in Dehydrated Foods

Of the numerous reasons for measuring and controlling the moisture content of dehydrated foods, one of the more important is the effect of moisture content on the stability of the food. The storage life of a food generally increases with decreasing amount of water and it is therefore customary to dehydrate the food to some "safe" moisture level which is determined empirically.

The propriety of using the moisture content of the whole food substance as the critical variable is, however, open to question because the moisture within a given food is not distributed uniformly. The total moisture content is a sum of the moisture contents of the various components, such as starch, pectin, protein, sugar, and cellulose.

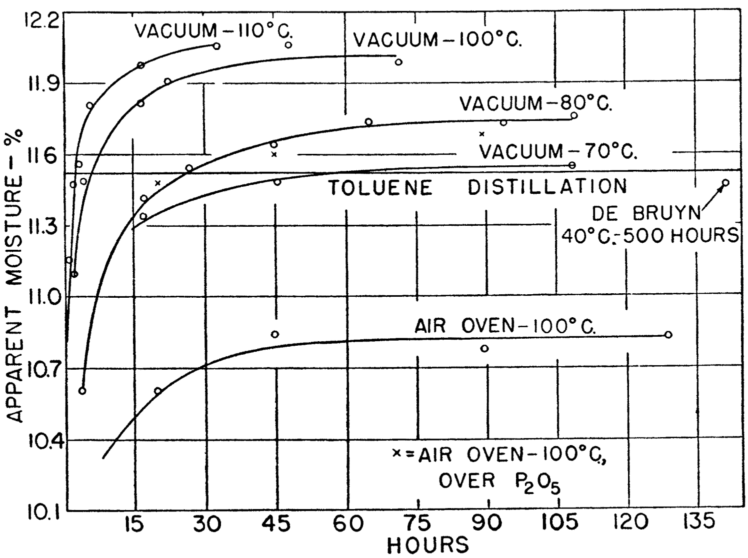


Figure 9. Determination of Moisture in Corn by Oven Methods (28)

The moisture content of a given component is in turn determined by the existing vapor pressure of water (or relative humidity) and is described by an isotherm for that component. Furthermore, it seems plausible to assume that the deterioration of the food is a function of the moisture content of the particular sensitive component (or components) that is involved in the deteriorative reaction. The over-all moisture content is thus of little significance.

Although we have no way of determining the moisture content of the individual components, it is not difficult to measure a property which is directly related to it and is common to all the components—the equilibrium pressure of water vapor. It seems logical, therefore, to use it as an index of moisture, as suggested by Makower and Myers (*20*).

The use of this index in dehydration has the following marked advantage. At a definite vapor pressure (and temperature) the equilibrium moisture content of a sensitive component is fixed, whereas the over-all moisture content varies with the composition of the food. Differences in composition in such foods as dehydrated vegetables or fruits are found among different varieties or result from different agronomic or processing conditions. Such differences reflect themselves in different "keeping quality" or "storage life" when comparisons are made on the conventional basis of equal over-all moisture content. It is very likely that these differences in behavior would be largely eliminated if vapor pressure were used as a standard of comparison, because equal vapor pressures would correspond to equal moistures in the sensitive components.

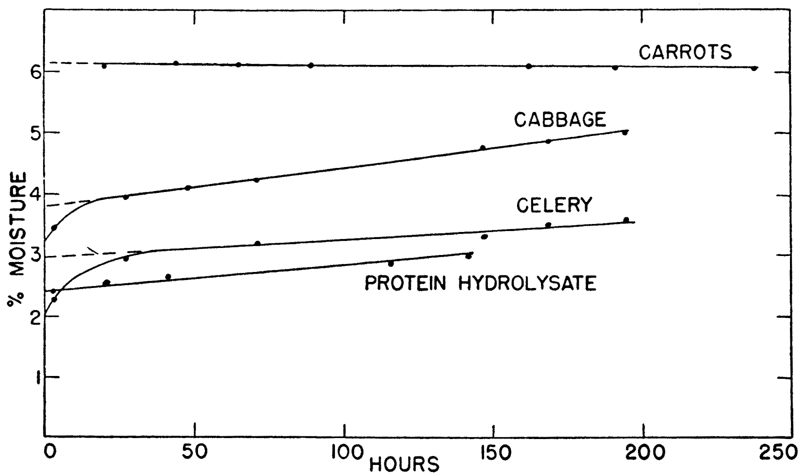


Figure 10. Moisture Determinations with Fischer Reagent

Intermittent titration method (*31*)

There are other advantages. Knowledge of vapor pressure would be exceedingly helpful in the design and control of drying equipment and in choosing packaging conditions for the product (*5*). In preparation of synthetic food mixtures, such as dry soup mixes, it would be possible to predict the transfer of moisture from one ingredient to another, from the knowledge of the vapor pressures of the individual ingredients. It is obvious that the safe moisture content of such a mixture corresponds to a vapor pressure of the most sensitive ingredient—namely, that ingredient whose safe vapor pressure is the lowest.

Measurement of vapor pressure is relatively simple and reproducible and has the outstanding advantages that the sample does not require weighing and the results do not depend on particle size (*20*). Fine grinding of a sample is, however, desirable to ensure adequate sampling and to promote equalization of vapor pressures when one is dealing with freshly dried substances. Makower and Myers (*20*) described a simple apparatus (with oil manometer) which could be used for measurement of pressures from zero to about 25 mm. of mercury with an accuracy of 0.1 mm. The time required for a measurement was 0.5 to 2 hours. A slight modification of this apparatus was used by Fischbach (*8*), and still another, employing a sensitive mercury manometer, was described by Legault, Makower, and Talburt (*14*).

There are, of course, occasions when vapor pressure data are not sufficient, and the moisture content must be known, as will be true, for example, in certain engineering cal-

culations or in evaluation of the product on a dry basis. Approximate values of the moisture content can be obtained from data on equilibrium moisture content (*8*, *19*, *37*), as illustrated in the isotherm for potatoes in Figure 2. One must remember, however, that a given class of foods cannot be accurately described by a single isotherm because of differences in composition.

Summary and Conclusions

The methods used for the determination of moisture in dehydrated foods have been divided into two classes, the direct and the indirect. The indirect methods, such as the electrical ones, must be calibrated in terms of the direct methods.

The most common direct methods are the oven, the distillation, and the Fischer methods. They can be made precise by careful standardization of the experimental procedures; their accuracy can be assured only by calibration against some accurate reference method.

Some of the common factors that control the accuracy of these direct methods are incomplete removal of water, thermal decomposition, volatility of nonaqueous components, and certain side reactions, such as oxidation and nonspecificity of the Fischer reagent.

A number of investigations describing different reference methods have been reviewed. They include drying to constant weight in vacuo at room temperature over a good desiccant, the redrying procedure (oven drying), the lyophilization procedure, distillation with organic solvents, the reversibility procedure (oven drying), and a modification of the Fischer method.

None of these reference methods can be given an unqualified recommendation as being the most practical and suitable for all foods. The room temperature drying method is prohibitively long. The redrying and reversibility procedures are rather laborious and time-consuming. The distillation method cannot ordinarily be used for calibration, because it does not usually yield a definite end point. The lyophilization procedure appears to be the most direct and convenient calibration method for materials, such as vegetables, that do not contain much nonaqueous volatile matter. For a material rich in these volatiles a modification of the procedure has been suggested whereby the volatiles could be isolated and determined separately.

The extraction of water at room temperature as a procedure for the calibration of the Fischer method requires further verification, because the reagent is not specific for water. It would be desirable to compare this calibration procedure with another. In general, agreement in the results of two or more independent calibration procedures might be used as a criterion of the attainment of accuracy.

It appears that each particular food must be investigated separately to determine the most advantageous and expeditious method of calibration. Furthermore, the accuracy of a given calibration method can be expressed only in reference to the particular food material under investigation and not for a method in general.

The choice of a particular method for routine measurements of moisture will depend on the nature of the food and the requirements of the analysis. The direct methods are generally more suitable where high precision or accuracy are desired and speed is not important. The indirect methods are usually applicable when speed is essential, but high precision is not required.

The advantages of vapor pressure as an index of moisture have been discussed. At the present time very few vapor pressure data are available for foods. It would be of great interest to measure vapor pressure concurrently with the moisture content in order to determine the usefulness of the vapor pressure in studies on the stability of dehydrated foods.

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch005

Measurement of Oxidative Rancidity

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Although rancidity is one of the most pressing problems confronting the modern food processor, there is no completely objective chemical test. Rancidity is determined ultimately by taste and smell, and stability is determined by placing the food in storage and allowing it to become rancid. However, accelerated tests save much valuable time and give useful information. The food chemist is making rapid progress in the prevention of rancidity and in improving the quality of fats and fatty foods.

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch006

One of the most prominent problems confronting the modern food processor is the prevention of rancidity. Rancidity affects not only the palatability of the food but the nutritive value as well—for example, oxidative spoilage of fats has been shown to be responsible for the partial destruction of the essential fatty acids (*5*) and of other dietary nutrients such as vitamins A (*12*) and E (*9*), and perhaps D (*26*) and certain members of the B complex (*5, 29, 30*). When one considers that nearly every food contains some fat and that this fat is subject to oxidative spoilage, the magnitude of the problem of rancidity is at once obvious.

Definition of Rancidity and Stability

The term "rancidity" has been employed in the past in both a general sense to indicate an off-flavor and in a specific sense, such as the hydrolytic spoilage of butter (*20*). Fats may develop off-flavors and odors through the absorption of odors, the action of enzymes, the action of microorganisms, and atmospheric oxidation. The present discussion limits the term rancidity to the development of objectionable odors and flavors through oxidative changes. Experimentally, this condition is found shortly following the end of the induction period—that is, if the oxygen uptake of a fat is plotted against time, an initial period will be found during which the rate of increase in oxygen uptake is low, after which the rate will increase in rather a sudden fashion, as indicated by a sudden rise in the curve. Shortly after this point is reached, the sample is found to be organoleptically rancid. The effect of an antioxidant is to lengthen the induction period; hence it delays the onset of rapid oxygen uptake.

"Stability" refers to a measure of the extent to which a fat resists the development of oxidative rancidity, and thus it is a measure of the induction period. Stability is usually expressed as the time that elapses under specified conditions before rancidity can be detected.

Such definitions imply that there is a reliable test for rancidity, but this is not the case. The difficulties are centered about the fact that in the final analysis, rancidity must be detected through organoleptic observation, which is subject to all the weaknesses inherent in a test involving personal judgment. As was pointed out in a recent discussion (*3*), these weaknesses include variabilities in the taste and odor sensitivities of persons in the same or different laboratories, their previous taste experiences, the prevailing condi-

In ANALYTICAL METHODS IN THE FOOD INDUSTRY; Advances in Chemistry; American Chemical Society: Washington, DC, 1950.

tion of health and taste apparatus, and the condition of the respiratory tract; all these factors lead to a lack of uniformity of judgment. No one chemical test has been devised which can measure and correlate accurately all the factors that act simultaneously to produce the odors and flavors called rancidity.

Determination of Active Oxygen or Peroxide Content

Of the many tests which have been submitted, the determination of active oxygen or peroxide content seems to give rather good correlation of data. During the oxidation of fat, certain oxygen-containing compounds are formed which are active in the sense that they are capable of liberating iodine from potassium iodide (*19*). The liberated iodine may be determined quantitatively and it thus becomes a measure of rancidity.

At this point, the problem of measurement of rancidity would appear to be relatively simple, in that one would merely determine the peroxide value at arbitrary intervals, and at a given point the sample would be considered rancid. This paper points out some of the difficulties involved in such a procedure from the standpoint of obtaining useful data.

First of all, rancid odors are due not to the peroxides themselves but to the breakdown products resulting in part from their action. The rate of breakdown depends in part upon the temperature, and therefore rancidity is detectable at various peroxide values depending upon the temperature of the tests. At refrigerator temperatures the peroxide value may be very high before sufficient breakdown occurs for organoleptic rancidity to be noted. A refrigerated sample may become organoleptically rancid very quickly when the temperature is raised, if oxidation has already occurred at the lower temperature; during accelerated tests, it has been noted that rancidity is detected at lower peroxide values than is the case when the same material is stored at room temperature. Certain antioxidants, perhaps because of their effects on rate of breakdown, will result in the detection of rancidity at peroxide values different from those considered normal for the unstabilized materials.

Another concept which is fundamental to the consideration of rancidity and to the problem of fat stabilization is that the oxidation is primarily a surface reaction. In other words, the surface of a food or of a packaged fat is the first to undergo deterioration, and peroxide values on the surface are therefore much higher than in the interior. Thus it is a faulty procedure to correlate peroxide value with rancidity by grinding or mixing the entire mass of a food or fat before determining the peroxide content. Such a procedure will result in the detection of rancidity at a lower peroxide value in a material with a relatively small surface than would be the case in the same material packaged in such a manner as to expose more surface, even though both samples are at the same state of rancidity as judged by organoleptic observation.

A number of methods have been proposed for the detection of rancidity. The determination of active oxygen consists of dissolving the fat in a suitable medium such as chloroform and acetic acid, adding potassium iodide, and titrating the liberated iodine with a standard thiosulfate solution (*16*, *20*). This is perhaps the most widely used method at the present time. Another procedure which has been proposed for the detection of peroxides employs ferrous ammonium sulfate and ammonium thiocyanate in acetone. The resulting red color of ferric thiocyanate is measured spectrophotometrically, and is said by the authors to yield more reproducible results than do the usual titration methods (*21*).

Detection of Aldehydic Substances

There are also methods based on the detection of aldehydic substances, for the typical odor and flavor of rancidity seem to be associated with the liberation of aldehydic materials during the oxidative deterioration. The Kreis test (*18*), perhaps the best known of these methods, consists of treating the fat with concentrated hydrochloric acid and a solution of phloroglucinol. The red color formed is attributed to a condensation product of epihydrin aldehyde with phloroglucinol. Historically, the chief difficulty with this method has been that fats which are not rancid will often give a positive Kreis test. It has been shown that if this test is quantitatively correlated with the induction period

the color intensity closely parallels the development of peroxides (*27*). The Schiff reaction for aldehydes (*28*) and the modified Schiff test (*31*) have also been used.

Another chemical test, "chlorophyll value," consists in titrating a standard chlorophyll solution with the fat in question and determining the end point by comparison with a standard under a mercury lamp. According to the author (*7*), the chlorophyll method is not dependent on corroboration by organoleptic tests, and is specifically applicable to the autoxidation of vegetable oils and to fats that have not been heated. Heat seems to develop fluorescing substances which interfere with the test. The principal objection to the chlorophyll value test is that one cannot make a random test of an unknown oil and say whether the oil is rancid or how soon it might be expected to become rancid. All these tests should be calibrated against odor and flavor in the particular type of fat or fatty food being studied, and no chemical test should be used as the only criterion of rancidity. These tests are simply useful adjuncts to the organoleptic tests, and are confirmatory in character.

Evaluation of Stability

The food processor and the food chemist are not so much interested in the detection of rancidity as they are in the evaluation of the stability of the fat or fatty food which they produce—that is, they want to know how long the food will remain in a nonrancid condition. The big problem here is that it is impossible to predict the conditions under which the food will be stored after it leaves the processor. Under ordinary conditions of storage, fats become rancid so slowly that stability information is virtually useless in the day-to-day operation of the plant. There is too much lapse of time between the making of improvements in plant operation and information as to the value of these improvements. An accelerated method of causing the development of rancidity is needed which will enable the laboratory to determine relative stabilities in a short period of time, and which will yield data that can be correlated with shelf life. There are, of course, a number of accelerated tests for the determination of fat stability, all basically similar in principle. Oxidation is accelerated under carefully controlled conditions while the original relations among the various samples are still preserved as far as possible until the time for spoilage has been reduced from months or weeks to days or hours. These tests utilize increase in temperatures, increased contact with air or oxygen, exposure to light or to catalysis by traces of metallic salts, etc. The progress of the test is followed by smell and taste and by the chemical tests described above. It is extremely important to calibrate all these methods with the particular type of product being studied.

The Schaal oven test (*27*) or some modification of it is perhaps the simplest accelerated test. The product being studied is placed in a warm oven (60° C.) and examined for indications of rancidity at various intervals. Disadvantages are lack of uniformity, due to the large personal errors, the difficulty of obtaining reproducible data, and the fact that a long time is necessary to obtain results. The personal errors may be offset to some extent by using large numbers of samples and plotting changes with time.

Another widely used test is the active oxygen method (*16*), in which carefully cleaned air is bubbled through the fat which is maintained at a constant temperature, usually about 99° C., although a recent modification employs a higher temperature (110° C.). The samples are tested organoleptically at hourly intervals and when a rancid odor is detected in the outgoing air, the peroxide value is determined chemically. This method has the disadvantage that the conditions are drastically different from those of ordinary storage, in that the air is continuously bubbled through the heated fat, so that equilibrium is maintained between the fat system and the environment. This is not the case in a packaged fat or other food. Moreover, rancidity is not detected at the same peroxide level in all fats (*3*, *4*).

Various methods have been suggested and used for the determination of stability, based on oxygen absorption. The fat is allowed to deteriorate in a closed system containing air or oxygen, and the amount of oxygen absorbed is plotted against time. Such methods are rather cumbersome and difficult to operate, although in recent years some very clever devices have been constructed which are almost automatic in operation (*10*,

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch006

11). One of these operates at constant pressure (*11*), while another utilizes the changes in pressure during oxidation to operate recording equipment (*10*). Such tests utilizing closed systems would need to take into account the possible liberation of volatile substances as a result of oxidation. Another adaptation of the above principle is the use of the Barcroft-Warburg constant-volume manometric apparatus, which has been applied to stability studies (*14, 15, 24*). Disadvantages are the time and apparatus involved, and the fact that organoleptic observations cannot be made without disrupting the experiment and that volatile products formed may interfere with manometric readings. Certain discrepancies between data on the Barcroft-Warburg and those obtained by the active oxygen method in studies of antioxidants have been traced to the effect of moisture in the environment (*23*).

Various tests for rancidity, such as the Kreis test, may be used in stability determinations if the intensity of reaction is plotted against time. The curves so obtained will give an indication of the length of the induction period. However, the plotting of a curve for each sample is somewhat laborious.

A common method for determining the stability of pastry, potato chips, and the like is to place a number of broken pieces of the product in 4-ounce mayonnaise jars with screw tops and store them at room temperature or in the Schaal oven in the absence of light. At regular intervals samples are removed and tested for peroxide content and organoleptic rancidity.

The correlation of accelerated tests with long age storage has been discussed by Thompson (*32*).

The final test of stability is, of course, obtained by shelf storage data, by placing the material under the conditions that will be encountered in commercial practice. But there are many problems encountered in the determination of shelf storage stability.

One of the factors that will affect the stability data is the temperature of storage; the higher the temperature, the shorter the time that will elapse before the fat becomes rancid.

Another factor requiring consideration is the presence or absence of light. Fats will develop off-flavors sooner in the presence of light than in the dark (*1*). It has also been observed that certain wave lengths of light will cause rancidity to develop in a shorter time than will other wave lengths (*8, 13*).

Type of Package

The type of package is important, not only from the standpoint of whether or not it will admit light, but if it does admit light, what wave lengths will be passed through or shut out. Then there are problems regarding the material from which the package is made. Certain metals will have a great influence on the development of rancidity. Copper and iron have a pronounced pro-oxidant effect. The permeability of the package to the fat is also of prime importance. If a package allows fat to ooze out onto the exterior surface of the package, the rancid odor will develop very quickly. Obviously, this does not mean that the entire mass of fat will become rancid at the same time as the surface layer, but the judgment of the consumer as to the freshness of the contents will be influenced by a rancid odor on the package.

Fat in a sealed package which is half filled has been shown to become rancid in a shorter time than fat in a full package (*6*). This is due to the presence of a smaller amount of air in the full package.

The presence or absence of oxygen in the package is of great importance. An inert atmosphere will cause higher stability figures to be obtained than if the atmosphere overlying the food is oxygen or contains oxygen.

The amount of air whipped into the fat is also of importance. Many processors of fatty materials include air or an inert gas in the fat as a means of obtaining a lighter product. This gas is intimately mixed with the fat particles, and, if oxygen is present, deterioration will proceed more rapidly than would be the case if the gas used were inert.

An important point is that we must assume that the technician is practicing meticulous cleanliness of all materials which come in contact with the fatty material to be

tested. It is useless to apply any stability test in the absence of extreme precautions as to cleanliness. This point is not sufficiently appreciated in many laboratories and processing plants.

Antioxidant Protection

One further problem should receive consideration in the present discussion, and is particularly important in current studies on the evaluation of antioxidants in the stabilization of fatty foods. Even though, by the use of one or more of the methods described here, it is possible to determine the stability of a fat, the stability data so obtained cannot be applied to the food products made from the fat. For example, one may find that a certain antioxidant is very effective in increasing the stability of a fat; but if one prepares pastries, potato chips, and the like by the use of fat containing this antioxidant, he will probably be disappointed to learn that the pastries and potato chips are not much more resistant to oxidation than when the antioxidant was not used. Usually, the stability of the fat before its use in cooking is of little value in predicting the stability of the pastry. There is as yet no adequate explanation for this lack of "carry-through" protection, but it is a very real problem to the food processor. Several recent discussions of antioxidants have dealt with this matter of carry-through, and have pointed out that most of the antioxidants now in use do not give protection to baked and fried products (*2*, *22*). One antioxidant which does give such carry-through protection is resin guaiac (*25*), a naturally occurring material. Another is butylated hydroxyanisole, a synthetic product (*17*). Both materials are in use at the present time.

Conclusions

There is no completely objective chemical test for rancidity. Rancidity is determined ultimately by taste and smell, and stability is determined ultimately by placing the food in storage and allowing it to become rancid. But numerous chemical tests can be applied by the experienced chemist as an aid to objectivity.

In spite of inadequacies in laboratory methods, much valuable time is saved through accelerated tests, and much valuable information is obtained. The foods chemist, through the judicious application of these tests, is making rapid progress in the prevention of rancidity, and in improving the quality of fats and fatty foods.

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch006

Measurement of Filth in Foods by Microanalytical Methods

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch007

The detection of filth in foods and the corroboration of insanitary conditions through microscopic identification of the indicia of filth constitute microanalytical problems of the first magnitude. The measurement and evaluation of filth elements in food products entail the use and integration of diverse types of knowledge, information, experience, and equipment. Sources for acquiring background and training are available to develop uniformity and soundness of judgment in the interpretation of findings by microanalytical procedures. Examples of some filth elements which have been found in certain foodstuffs illustrate methods of isolation, means of recognition, and the significance of evidence developed upon microanalysis.

The logical opening for a discussion of this type is a definition of the term "filth" from the regulatory standpoint. Filth is that which is repugnant to the normal individual when that individual has full knowledge of the character of the contaminant that is classed as filthy. For example, to bite down on a hard raisin is one thing, but to encounter a hard substance which one knows to be a pellet of rodent excreta is an entirely different and disgusting experience. Application of this definition determines whether contaminants present in food should be classed as filth or as foreign matter. Under the Food, Drug, and Cosmetic Act there is no requirement that either be demonstrated to be injurious to health; however, the law does make the positive requirement that foods be free from such contaminants. The law also makes the positive requirement that foods should not be produced under insanitary conditions whereby they may be contaminated with filth. The word "may" in this connection is to be given its usual significance. The finding of filth in foods in most instances represents carelessness somewhere along the production, distribution, or storage line. Microanalytical methods, therefore, in a regulatory sense, are used to determine, first, if a food is illegal within the meaning of the statute because of the presence of filth therein and, secondly, if the filth elements found in a food constitute an index of filth pickup as a result of insanitary practices at point of production or point of storage.

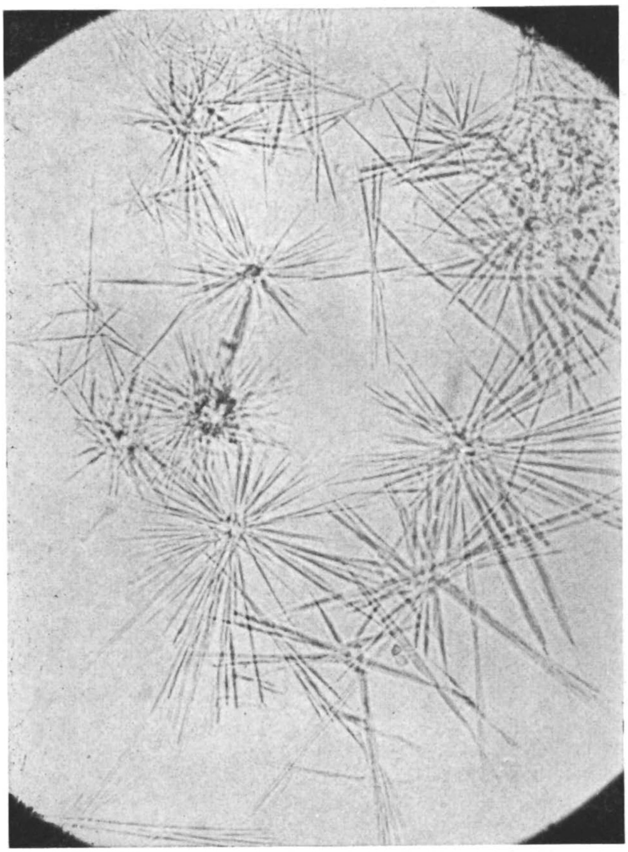
The final measurement of filth by microanalytical methods entails in nearly every instance a series of manipulations which in general are based on diverse types of information—chemical, physical, and biological—to be interpreted through educational background, experience, and judgment. Because the microscopy of foods deals largely with the identification of both normal and adulterant ingredients, it is incumbent upon the analyst to ascertain to the best of his ability the characteristics of the matter with which he is dealing. The methods employed are generally based on direct microscopical obser-

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vations which give the answer as to the identity of substances often unobtainable by other means. The actual procedures and manipulations of the methods are in most instances simple, but the technical dexterity required constitutes a minor part of the skills necessary for efficient, accurate, and discriminating microanalytical analyses. The major work begins after the filth elements have been isolated.

Significance of Filth Sources

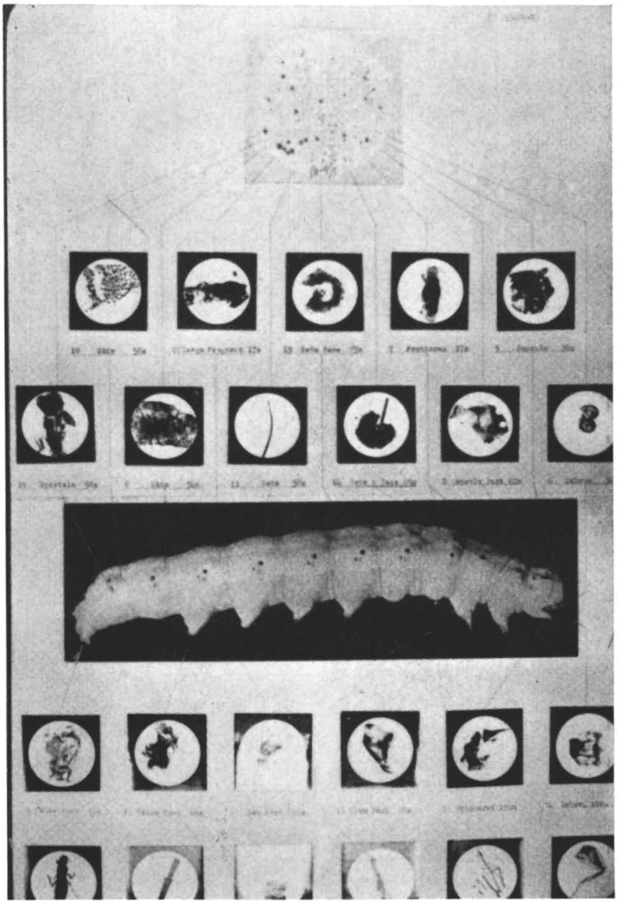
Sources of filth and contamination are diverse and numerous, each contributing its individual weight to the final summation and measurement. Rats, mice, and flies are themselves filthy in habit and also indicative of filthy conditions. Any evidence of their presence in or about a food product constitutes a heavy measure of filth. Insects which infest foodstuffs, or which live in or close to a food processing plant, create and leave evidence of their presence and reflect field, factory, and storage conditions.



Crystals Produced in Xanthydrol Test for Urea (360×)

A knowledge of insect habits, habitat, and life cycles is essential in any attempt to measure the significance of the filth contributed or indicated by them. The various insect forms, eggs, cast skins, excrement, webbing, and tunneling are all factors denoting the duration of insect life in, on, or near food products in the different stages of their production, manufacture, or storage. Maggots in fruits, vegetables, or mushrooms reflect different conditions of factory sanitation, horticultural care, or growth. Cockroach parts and excrement, feather barbules, or bat hairs and excrement may indicate improper

storage, or poor orchard and farm sanitation. Barnyard manure fragments must be differentiated from straw fragments before they can be measured as filth elements. Under the modern methods of production, a relatively large volume of a dairy product need show only a few manure fragments to indicate a heavy measure of original filth. Ants, spiders, mites, sow bugs, and psocids bear their own significance aside from the esthetic, depending on the manufacturing, storage, or natural conditions involved.



Corn-Ear Worm *(Center)* and Parts Recovered from Comminuted Tomato Product

Above. **Skin, large fragment, seta base, prothorax, capsule, spiracle, skin, seta, seta and base, capsule part, labrum**
Below. **False foot, false foot, leg part, true foot, spinneret, labium**

There can be no set standard or measurement for all types of filth and filth elements, nor can any one procedure be employed, any one set of circumstances considered, or any one kind of evaluation applied. In any case, however, it is of prime importance that a thorough study of method precede manipulation; furthermore, it is highly desirable that the operator have sufficient knowledge of production and storage conditions to draw logical and supportable conclusions from the "filth elements" recorded. Obviously, degree of infestation or rot could not be expected to follow with mathematical exactitude the constant curves found in other sciences.

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Methods and Means of Analysis and Interpretation

The methods employed for microanalysis of food and drug products have been grouped into three categories: (1) biological microscopical, (2) microscopical chemical, and (3) microscopical petrographic. Biological materials (plant and animal products) are identified by their characteristic morphological, histological, or cellular structure; amorphous chemical ingredients by microscopical appearance of characteristic precipitates, or specific color reactions on treatment with special reagents; and crystalline substance by means of diagnostic optical constants measurable with the aid of the polarizing microscope. The greater part of the routine work of microanalysis, as far as food products are concerned, falls into the first category and makes use of the full resources of low-power and compound microscopes. The jeweler's loop, or a hand lens, about 4× magnification, is convenient and suitable for macroscopic observations. The wide-field, Greenough-type binocular microscope, which may be used for the examination of filter papers at a magnification of about 25× or for identification of relatively gross contamination, is also useful for rot fragment counting and some verification work, at a magnification of 40 to 50×. The compound microscope is indispensable for identification of

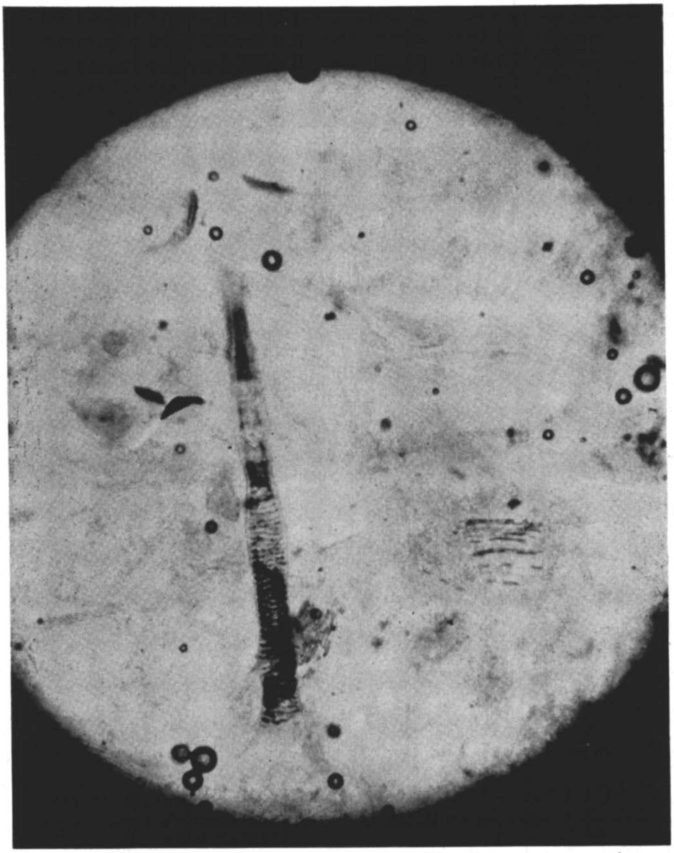


Mold Filament Entering Tomato Cell (360×)

hair, mold, yeasts, spores, bacteria, and insect and worm fragments. The petrographic microscope finds its greatest use in the identification of crystals and starches. The phase microscope has been used on bacteria and in tissue work, but its possibilities for other specimens are yet to be determined.

We have, in the Howard procedure and technique, a reliable microanalytical method

for measuring rot caused by mold, yeasts, spores, and bacteria in fruit and vegetable material. At the same time it serves as a means for detecting in the finished product sources of contamination and spoilage, the cause of certain conditions of rot, and, frequently, factory practice, control, and sanitation.



Rat Hair Fragment in Pellet of Rat Excrement (135×)

Showing compound medulla and internode

In order to establish a sound basis for obtaining comparable and reliable results among the many microanalysts within the Federal Government concerned with evaluating microscopical findings in food products, the microanalytical division of the Food and Drug Administration in 1944 developed two manuals (*3*, *4*), dealing with microanalytical procedures for the examination of food and drug products and with photomicrographs. The manual of methods included all the procedures then in use by the administration for the examination of such products. Most of the methods appearing in this manual have been further edited (*1*). Also, in 1944, in conjunction with the manual of methods, and as an extension of it, a circular was published (*2*), making available to industry discussions of what the analyst observes microscopically in the food (or drug) product, compared with authentic material and directed toward developing a background for evaluating results of microanalyses. Through these publications and subsequent revisions there are in use standardized procedures for the examination of food products, eliminating variables resulting in analysis by entirely different methods and discrepancies that might result from the analysis of the same food product by trained and untrained analysts, both using the same technique.

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The following examples serve to illustrate certain situations wherein filth elements may enter foodstuffs, the means of isolating and verifying these contaminants, and their possible significance.

Rodent and Insect Contamination

Filthy conditions, due to rodent or insect population, may be observed in a grain warehouse. The inspector notes rat-chewed flour sacks and sacks contaminated with rat excrement. He removes a sample of sacking and flour from such contaminated areas and submits them to the analyst. Urine fluoresces under ultraviolet light. Where rodent urine is to be confirmed, the xanthydrol test is one of several that may be used. Excreta pellets may be moistened with water or an appropriate clearing solution and crushed for observation under the compound microscope. The presence of striated hair fragments indicates rodent excrement.

When such contaminated flour enters trade channels, either as such or in the form of prepared products, the finding of hair fragments is highly significant. Whether they may have come from the flour or have originated in a bakery or factory does not lessen their repugnance to the consumer. Recovered hairs are identified by means of their several characteristics. Some of the more salient are: conspicuous internodes and compound medulla of rat hairs, inconspicuous internodes and smooth continuous medulla of cat hair, and differences discernible after treatment with 10% sodium hydroxide and after treatment with hypochlorite.

If flour or meal has become contaminated with storage insects after milling, the insect parts or larvae may be removed for identification by sieving or by a flotation procedure, but perhaps only excrement remains in the sample. This is about the same color as the material upon which the insects have fed and has generally the same appearance macroscopically. By means of the fluorescent light, however, pellets may be rendered more readily visible. If such flour is treated with clove oil, the pellets stand out distinctly and may be readily counted.

For purposes of interpretation it may be desirable to know the relative size and number of insect parts in contaminated flour, meal, or bakery goods. Particles which pass through a No. 140 sieve (150 meshes per inch) can be identified. These may be recovered from flour with gasoline, after acid hydrolysis, using the Wildman trap flask.

Worm, Fly, and Mold Damage

To illustrate how some filth elements may be detected, measured, and evaluated in fruit and vegetable products, let us consider tomatoes, which are subject to at least three types of depredation: worms, flies, and mold.

The corn-ear worm and the pinworm are probably the most prevalent of the worm pests which attack tomatoes. Their various parts may be found in a finished product to indicate the use of raw material infested by them.

Contamination by flies may occur directly in a factory, especially if material is allowed to stand unprotected for long periods while being held for processing. Failure to dispose of refuse promptly, particularly outside the plant, frequently creates ideal breeding and feeding places for these pests. Under such circumstances, parts of the adult vinegar fly, its larvae, or eggs, when found in the comminuted product, provide definite evidence of careless and insanitary operations.

The diseases which cause tomatoes to rot are numerous, but let us consider those produced by mold alone, and as grouped under that general heading. A few of the more common molds that may attack tomatoes are: *Penicillium*, *Fusarium*, *Rhizopus*, *Aspergillus*, *Rhizoctonia*, *Alternaria*, and the slime mold *Oospora*, which latter may also be found on dirty or insufficiently cleaned factory machinery. Mold filaments can be stained for more detailed observation. (The author personally prefers Poirer's blue stain and clearing solution—lactophenol with cotton blue—because of its effective clearing powers in addition to its staining properties which are to some extent selective for mold. Gentian violet is the stain used in the rot fragment and butter mold count.)

Mold type of spoilage is directly measurable by means of the Howard mold counting

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch007

technique, which may also detect the presence of yeasts. They are best studied after staining, however, and at a magnification higher than is usually used for mold counting.

The presence of actual clumps of mold rot in a comminuted product can be demonstrated by use of the rot fragment count, which employs a staining technique and observation at about 50× magnification.

Summary

The measurement of filth elements by microanalysis is a valuable adjunct in the enforcement of the Food, Drug, and Cosmetic Act and serves as an efficient means of evaluating conditions of cleanliness, decency, and sanitation in food-producing plants. This, of course, is in addition to the value of microanalytical methods in the determination of the fitness of foods as they reach the consumer. The techniques available, together with proficiency of manipulation, repeated references to authentic materials, and sound judgment in the interpretation of results, provide effective enforcement weapons in the constant war to prevent the production and interstate distribution of products which are unfit for the table of the American consumer.

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch007

Some Aspects of Control Methods in the Canned Food Industry

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch008

Although notable improvements have been made in methods used by the control laboratories serving the canning industry, a great deal of room remains for further improvement. A survey of some of the functions of control laboratories demonstrates the need for better methods of determining spray residues, traces of metals, vitamins, moisture, and head-space gases. As is the case in other industries, improved instrumentation offers a solution to some of the existing problems.

Because the quality and health aspects of foods cannot be measured by a single index, it necessarily follows that the subject of control methods in the canned food industry is very broad, and includes chemical, physical, organoleptic, and bacteriological tests, only the first of which is discussed here. The measurement of color, odor, optical clarity, texture, viscosity, and chemical composition has been used to evaluate canned foods, but in many cases the methods that are applicable to one product are either not applicable to another, or can be used only after considerable modification.

Cannery control laboratories have greatly expanded in their number and size during recent years (*1*), and have become indispensable to those canners who market their products under a single label and on a nationwide scale. Although a review of the methods which have been used successfully in control work would make an interesting subject for discussion, the primary purpose of this presentation is not so much to review established procedures as to discuss a number of methods that are deserving of further attention. In addition, recent developments in analytical methods and instrumentation are discussed from the standpoint of their application to canning problems.

Before discussing the problems of control laboratories, it is important to have a clear picture of the revolutionary changes which have been, and still are, taking place in the field of analytical chemistry. One has only to observe the recent issues of *Analytical Chemistry*, noting especially the editorials of Murphy (*14*) and the articles of Müller (*13*), to recognize the trend away from the classical gravimetric and volumetric methods of 20 years ago.

Instrumentation is the theme of modern analytical procedures and has also been the roots supporting the growth of control laboratories. It has given the control laboratories tools which have made possible the collection of data in a time short enough to permit the action suggested by the data. It has made available methods that are operable by semitechnical help, but at the same time it has increased the responsibility of those who are charged with selecting control methods and supervising analytical work.

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Advances in Chemistry; American Chemical Society: Washington, DC, 1950.

Control laboratories in the canned food industry are usually divorced from the research organization to a lesser degree than is the case in the chemical and allied industries. For this reason, a closer relationship exists between the problems of the control laboratory and the research laboratory. Although from a research standpoint this condition is often considered undesirable, it has considerable merit in the case of the canned food industry, in which production may be seasonal and often of rather short duration. The collection of control data in many instances may also serve for research purposes—for example, in the case of soil analyses, which may be correlated with agricultural research designed to improve crop yields. Because the variables which affect the quality of canned foods must usually be investigated rather extensively, and often over a period of more than one year, the application of statistical methods to data collected for control purposes can conceivably make a substantial contribution to a research program.

The interval between the development of an analytical procedure and its adoption as a control method is an important one. Tests of new methods during this period are exacting in their demands for a satisfactory combination of simplicity, speed, and accuracy. Much credit for adapting analytical methods to general laboratory use is due to such organizations as the Association of Official Agricultural Chemists, the American Oil Chemists' Society, the American Association of Cereal Chemists, and the Association of Vitamin Chemists. However, these organizations are often chiefly concerned with the application of a procedure as a referee method. Consequently, the responsibility for selecting and adapting a control method to cannery control purposes is usually an important function of the individual responsible for quality control.

Determination of Spray Residues

The presence of toxic spray residues, such as lead and arsenic in fruits for which lead arsenate is used as an insecticide, has, in the past, received considerable attention from both the growers and the canners of certain fruits and from state and federal regulatory authorities. The latter have established specific tolerances for these elements as residues in certain fresh fruits, and although the tolerances do not apply specifically to canned fruits, canners have generally accepted the food and drug tolerance or the more rigorous tolerances suggested by the American Medical Association. Because, in the case of lead arsenate spray residues, the substances being determined are inorganic in nature, the analytical procedures [usually the A.O.A.C. Gutzeit or Cassil methods for arsenic (*2*) and the dithizone method (*19*) for lead] are capable of good precision and the results lend themselves to a clear interpretation, regardless of whether or not chemical reactions occur between the residue and the canned product. A different situation exists in the case of many of the new organic insecticides and herbicides which have been introduced during recent years, and which are rapidly gaining popularity with growers of fruits and vegetables for the canning industry.

The development of analytical procedures for the determination of traces of these materials, of which DDT is at present the most prominent, has presented a real challenge to analysts interested in control methods suited to the food industry. The problem of developing suitable methods is doubly complicated by the fact that we have only a meager knowledge of the chemical changes which may occur when these materials undergo heat processing and storage.

In addition to their concern regarding spray residues of the lead arsenate and organic types, canners whose fruits are sprayed with lime sulfur have reason for concern over the presence of sulfur in their canned products. The presence of elemental sulfur or simple compounds of reduced sulfur in canned foods, especially in those products having an acid character, is objectionable, not only because it may be the source of sulfide flavor and staining, but also because it may actively accelerate the formation of hydrogen by the corrosive action of the product on the container.

That sulfur may be introduced into foods for canning by the use of sulfur-

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch008

bearing insecticides has been demonstrated by Clough (*7*), who points out that the use of lime sulfur for controlling insect infestation of gooseberries may result in hydrogen springer formation of the cans during storage. As a result of sulfur residues, canners have suffered substantial losses from both hydrogen springer formation and off-flavors of their product.

Usually the qualitative detection of sulfur in extremely small amounts (less than 1 p.p.m.) can be made readily by observing the presence of the characteristic black sulfide stain, which is formed on the container walls when unenameled tin plate containers are used. In the past, the use of sulfur-bearing insecticides has been confined to lime sulfur mixtures, and quantitative analytical methods have only occasionally been applied for control purposes. Recent years have witnessed the introduction of several organic insecticides, such as fermate (iron salt of dimethyldithiocarbamic acid), and zerlate (zinc salt of dimethyldithiocarbamic acid), which contain organically bound sulfur. At the present time, little is known concerning the tendency of these materials to be carried over into the final product and the subsequent effects they may produce. It is not unreasonable to assume that organic spray residues of the type mentioned will, to a large extent, resemble lime sulfur in so far as their actions on the container and product are concerned. It is not unlikely that in the near future a real need will exist for quantitative methods for determining these materials in the control laboratories.

Determination of Trace Metals. As exemplified by the black sulfide discoloration caused by the presence of minute amounts of copper in corn, by the turbidity caused by minute quantities of tin and iron in beer, and by the potential toxicity of arsenic and lead, which may be introduced in the form of spray residues, methods for the determination of traces of metals in the presence of an overwhelming amount of organic material are of primary concern to some cannery control laboratories. Although the use of polarographic and spectrographic methods is expanding, and one or the other of these instruments is a practical necessity in the case of the determination of minute amounts of tin, by far the greater portion of trace metal determinations are made by spectrophotometric methods. Usually, it is necessary to destroy the organic matter before determining the inorganic constituents, but the determination of iron in beer is an exception to the rule. Ignition of the dried samples in a muffle furnace or digestion of the samples in a hot oxidizing acid is usually employed for this purpose, but neither of these operations is without its disadvantages. In the case of dry ashing, care must be taken that the constituents being determined are not volatilized during the ignition, and, probably more important, that they are not fused to the ignition vessel. In the case of digestion with oxidizing acids, the purity of the acid used becomes especially important, for the amount of the acid may exceed by severalfold the weight of samples taken.

Previous to the publication of polarographic methods by Lingane (*10*) and Alexander (*8*) in 1945 and 1946, spectrographic methods were the only reliable techniques for determining minute quantities of tin (*6*). In somewhat larger amounts, however, tin in foods may be determined by iodometric titration (*2*). Because of their high sensitivity in producing colored reaction products, organic reagents are used extensively for the determination of traces of metals. Lead, for example, is almost exclusively determined using dithizone (*19*), copper using dithizone (*4*) (diphenylthiocarbazone) or diethyldithiocarbamate, and iron with either *o*-phenanthroline (*11*) or 2,2′-bipyridine (*12*).

Regardless of whether polarographic, spectrographic, or color methods are used for the determination of trace metals, great care must be exercised to avoid contamination of the sample during its preparation and during the course of analysis. Control chemists wishing to review the fundamentals of methods for determining traces of metals can gain much from Sandell's excellent treatment of the subject (*17*).

Determination of Vitamins

Although reliable methods (*3*) for the determination of ascorbic acid, thiamine,

riboflavin, niacin, vitamin A, and carotene have been developed and used extensively in research work and in surveys of the nutritive value of canned foods, comparatively few cannery control laboratories include vitamin determinations as a regular part of their activities. With the possible exception of the relatively simple determination of vitamin C in some products by the dichlorophenol-indophenol titration procedures, there is undoubtedly a great need for a simplification of vitamin methods so as to make them better suited for control purposes.

Retention of vitamin C during the canning of tomato and citrus juices, which are excellent carriers of vitamin C, has been studied extensively by numerous workers, and that these products retain ascorbic acid to a high degree during storage in cans has been established. In the case of tomato juice, however, an appreciable amount of destruction of vitamin C may occur as a result of oxidation during the several stages of preparation. In this connection, the simple polarographic technique described by Lewis and McKenzie (*9*) for the rapid determination of dissolved oxygen in fruit juices should deserve the close attention of control laboratories interested in lowering the dissolved oxygen content of fruit juices as a means of improving the retention of vitamin C.

Organoleptic Tests. An important function of cannery control laboratories is that of making organoleptic tests. Although organoleptic tests have obvious disadvantages in that they involve a great deal of "personnel element," in many instances qualitative and semiquantitative determinations can be made only in this manner. The training of tasters and the organization of taste panels have received considerable attention in recent years (*16*).

Moisture Determinations. Because the moisture content of many fruits and vegetables is an index to maturity and quality, moisture determinations are employed extensively in the grading of raw fruits and vegetables, and in some cases—for example, tomato products—the finished item may be sold on the basis of moisture content.

Refractive index in the case of corn and tomato products and specific gravity in the case of tomato products have been used extensively as an indirect means for determining moisture; the A.O.A.C. vacuum drying method is usually taken as the standard for calibration. Although electrical instruments operating on the principle that the capacity of a condenser having fixed plates can be calibrated in terms of the moisture content of the material between the plates have been used extensively for the measurement of moisture in relatively dry products such as grains and cereals, this principle has only recently been applied to products of high moisture content (*18*). In the latter case, the comminuted sample is diluted with a relatively large volume of an organic solvent having a low dielectric constant, and advantage is taken of the fact that the presence of a small amount of water having a high dielectric constant will effect a large increase in the dielectric constant of the mixture as compared to that of the organic solvent. In the authors' opinion, this method is deserving of extensive investigation, inasmuch as it conceivably could be applied to a wide variety of products.

Gas Analyses. Following the introduction of hermetically sealed cans for packaging of dry or relatively dry products such as coffee and vegetable shortening, it was recognized that the quality of these products can be preserved for a longer time if they are retained in an atmosphere that is substantially free from oxygen. In accordance with the desire to package such products in an absence of oxygen, highly efficient equipment for sealing containers in a high vacuum or for sealing them immediately after flushing with an inert gas such as nitrogen has been developed. Chemical methods have been used by the packers of these products for evaluating the degree of oxygen removal (*5*). In the case of vacuum-packed coffee, it might be supposed that a simple vacuum determination using the puncturing-type vacuum gage commonly used in many branches of the canning industry would be an adequate index to the degree of oxygen removal. Freshly ground and roasted coffee, however, contains an appreciable amount of carbon dioxide within its cellular struc-

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch008

ture. The carbon dioxide begins to diffuse from the coffee immediately after closing. Furthermore, the oxygen that may be sealed into the can at the time of closure is depleted rapidly as a result of its reaction with the coffee. The extent of oxygen removal at the time of closure, however, has been determined, even after the cans have been in storage for long periods, by determining the nitrogen content, correcting empirically for a small amount of nitrogen desorbed from the coffee, and calculating the amount of oxygen as one fourth that of the nitrogen.

The usual Orsat techniques are adequate for the determinations, but special equipment is desirable for puncturing the containers and taking the gas samples (*5*).

Orsat techniques are also adequate for determining the efficiency of oxygen removal when head-space flushing with nitrogen or carbon dioxide is employed for removing the oxygen from shortening cans. The amount of sample in the head space of water-packed fruits and vegetables is usually not adequate for the Orsat technique, but sufficient if Van Slyke manometric methods are used. Although the latter types of determinations are not usually made by control laboratories, there are instances in connection with hydrogen springer formation and microbiological spoilage where the analyses of head-space gases may be of importance.

Instrumentation. Admittedly, it is difficult to distinguish clearly the difference between an analytical procedure involving instrumentation and a procedure in which instrumentation is not involved. Even such relatively simple procedures as the gravimetric determination of lead by precipitation as lead sulfate require a fairly complex precision instrument—an analytical balance. Perhaps the most distinguishing feature of recent developments in instrumentation is the fact that the property being determined—for example, mass or concentration—is measured indirectly by taking advantage of a series of physical properties which can be made to vary in a predetermined manner, depending upon the amount or concentration of the substance being determined.

Recent spectacular developments in electronic and optical instruments, which have resulted in such highly practical control equipment as automatic recording spectrophotometers and spectrographs, and automatic pH and temperature recorders and controllers, reflect the ability of instrument designers to convert one type of phenomenon readily to another. For example, it is a relatively simple matter to adapt a minute physical movement so that it will control a light beam which, in turn, will generate or control a voltage which, with suitable amplification, can be made to regulate a great variety of electrical and mechanical equipment almost without regard for the energy requirements of the latter.

Cannery control laboratories have not generally adopted the more complicated instruments to an extent comparable to their use in control laboratories associated with the chemical industries. The use of vacuum tube voltmeters for pH and temperature measurements and spectrophotometers for color measurements seems to point the way to further expansion in this direction. Instrument manufacturers and suppliers are usually anxious to learn of new problems, and control chemists may profit by bringing their problems to their attention, even though a solution may appear remote. The newer optical, electrical, and electronic instruments employ the principles of physics to a far greater degree than they apply chemical principles. This fact is a major concern of those who have discussed the subject (*15*), and who have pointed out that in many instances chemists lack the sufficient background in physics which is necessary to good understanding of modern methods of analysis.

Miscellaneous Determinations. Several other problems are deserving of brief mention. The citrus industry, for example, would welcome an improved and more rapid method for determining volatile oil in citrus products. The tomato canning industry could profit by more rapid and accurate methods for determining tomato solids, and also by a method that would predict the increase in viscosity which takes place during concentration.

The determination of alcohol-insoluble solids as a means for grading peas can

Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch008

hardly be called satisfactory for control purposes. The fruit canning industry has need for a continuous or recording method for measuring sugar concentrations, perhaps in the form of a recording refractometer. Simple methods for measuring the color of fruits and vegetables are far from adequate, and equipment for more reliable measurements is complicated and expensive. Methods for measuring color, flavor, and odor are almost nonexistent, except in a few special cases, such as saltiness, where the flavor is related to a simple constituent. A more rapid and convenient method is needed for ascorbic acid, particularly if it is desired that the canned product contain certain minimum levels of this vitamin.

Acknowledgment

The authors wish to express their appreciation to L. E. Clifcorn in particular and to the several members of the Product and Process Research Division and the Customer Research Division of Continental Can Company, Research Department, for helpful suggestions in the preparation of this paper.

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Publication Date: June 17, 1950 | doi: 10.1021/ba-1950-0003.ch008